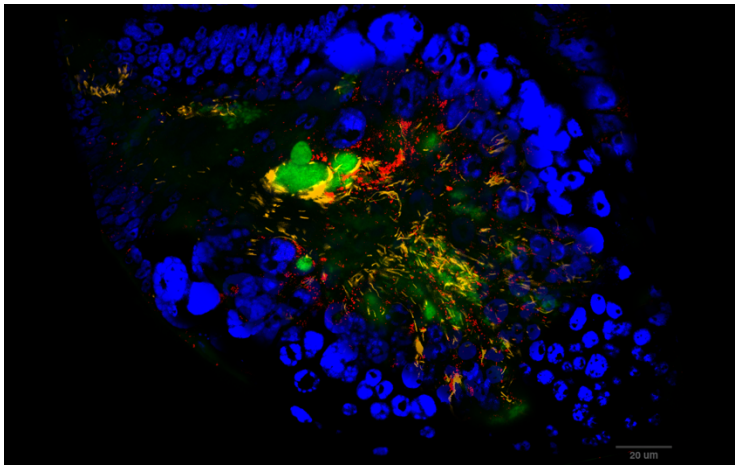
UNIVERSITY OF LIVERPOOL

Institute of Infection, Veterinary
and Ecological Sciences

Torix *Rickettsia*: aspects of diversity, host range and symbiont-host interaction.



Thesis submitted in accordance with the requirements of
the University of Liverpool for the degree of Doctor in Philosophy

Panupong Thongprem

December 2020

DECLARATION

I hereby declare that the work in this thesis is based on research carried out at the Institute of Infection, Veterinary and Ecological Sciences, University of Liverpool. The work contained in this thesis is my own, unless otherwise acknowledged and cited. The data that have already been published in scientific journals are specifically declared in each chapter with authors contribution described. This thesis is original and has not been previously submitted for any other degree.

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ABSTRACT

Rickettsia bacteria have traditionally been considered as the aetiologic agent of deadly arthropod-borne diseases in humans and livestock. However, more recent studies have discovered *Rickettsia* as non-vertebrate pathogens that are actually important to invertebrate evolution as symbionts. Recently, *Rickettsia* in the 'torix' clade were described from glossiphoniid leeches. This clade has since been observed to infect a wide range of invertebrate species and is thought to be most common in host species associated with freshwater habitats. This leads to a general hypothesis that torix *Rickettsia* are a common endosymbiont of freshwater taxa. However, this hypothesis is yet to be formally tested. To assess this hypothesis, I firstly investigated in-depth a freshwater-associated insect order, the Odonata (dragonflies and damselflies), in which torix *Rickettsia* had not been previously recorded. This study revealed the first incidence of torix *Rickettsia* in odonates, present in roughly 10% of the screened species. Maternal transmission of this endosymbiont was observed in a damselfly (*Coenagrion puella*), and this strain has likely driven mtDNA introgression between the insect and its sister species (*C. pulchellum*). Then, I expanded the screen to test for torix *Rickettsia* in other invertebrate taxa and compared the infection frequency between freshwater and terrestrial communities. Fisher's exact test indicated that the proportions of infected species from freshwater community is significantly higher than the terrestrial group in three representative insect orders. In addition to this broad screen, torix *Rickettsia* in a few blood-feeding insects are recorded for the first time, including mosquitos (*Anopheles plumbeus*), black flies (*Simulium aureum*) and the common bed bug (*Cimex lectularius*). Bed bugs were then established as a model system to study biological impacts of torix *Rickettsia* carriage. Symbionts in the bed bug were transmitted via matrilines only. There were no signs of reproductive parasitism, sex ratio distortion or cytoplasmic incompatibility phenotypes. Torix *Rickettsia* only express mild parasitic impacts on *C. lectularius* biology by slowing development time and reducing fecundity. Finally, this thesis raises three questions for onward study; i) why torix *Rickettsia* are abundant in freshwater biomes, ii) how do torix strains transition into terrestrial species and iii) how torix *Rickettsia* are associated with broad spectrum of eukaryotic hosts. Possible scenarios for these three questions are discussed for future study.

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CHAPTER 1

Introduction

1.1 Heritable endosymbionts

Studies of the biology of animals, plants and fungi in recent decades have recognised the importance of including microbial symbionts as part of individual biology, and by extension, their importance in treatments of ecology and evolution. Many aspects of biology – from digestion through to defence against infection – are impacted by microbial partners, and as a consequence many of these bacteria are also involved in the adaptive process as a co-evolutionary partner [1]. The nature of the interactions is diverse. In evolutionary terms, some symbiotic microbes are deleterious to the fitness of their host. Other multicellular organisms can survive without microbes but are less fit compared to when microbes are associated. Finally, some organisms need the microbial symbiosis to survive, they are obligately dependent [2].

In many invertebrate species, and in plants and fungi, symbiotic microbes may transmit vertically, from a female to her offspring. Often existing for millions of years as host associated heritable microbes, they have adapted to live as endosymbiont inside host cells. This adaptation commonly limits the full functional metabolic capabilities of the microbes (by reducing the genome size 2 to 4 times smaller when compared to their relative free-living form [3, 4]) to be a strictly obligate endosymbiotic form. That is, the bacteria lose their capacity to reproduce outside the host cells. Heritable microbes, with their tiny genomes commonly retain only the genes essential to maintain their core physiology and cell replication capacity, and the biochemical synthesis associated with symbiosis with the host [4].

1.1.1 Primary and secondary endosymbionts

Some eukaryotic organisms cannot survival independently of their symbionts due to their limited metabolic capability. They thereby require a symbiotic partner as an endosymbiont to supplement some nutritional compounds or biochemical molecules for their development and survival. These endosymbionts will be obligate themselves, live inside the host cells as a ‘primary endosymbiont’ (P-endosymbiont), which enable their host to inhabit otherwise inaccessible niches. There are many examples of these primary endosymbiont/host interactions e.g., the

pea aphid (*Acyrthosiphon pisum*) and its obligate bacteria *Buchnera* [5], the common bed bug (*Cimex lectularius*) and its endosymbiont *Wolbachia* [6], the carpenter ant (*Campotonus floridanus*) with the endosymbiont *Blochmannia* [7], and the tsetse fly (*Glossina morsitans*) with its endosymbiont *Wigglesworthia glossinidia* [1]. This host-symbiont relationship has formed through co-evolutionary process over several million years, such that neither the host nor symbiont are viable without one another. In this case, it is common to see that the host provides a special organ to harbour their primary endosymbionts, i.e., 'bacteriome'. The bacteriome (or previously called 'mycetome') comprise lots of bacteriocytes that are infected with high numbers of endosymbionts. The locations of these organs are varied, e.g., in digestive tracts [8, 9], abdominal cavity [6, 10, 11], and salivary glands [8], depending on the host species and the service function of the symbionts.

In contrast to these mutually dependent interactions, there are other endosymbionts that facultatively live inside host cells but do not form the obligate mutualistic relationship. These bacteria exist as a 'secondary endosymbiont' (S-endosymbiont) in which the host is viable in the absence of them. Examples of these are *Rickettsia* endosymbiont in the pea aphid (*A. pisum*) [12], γ-proteobacteria (BEV-like symbiont) in the common bed bug (*C. lectularius*) [6] and *Sodalis* endosymbiont in the tsetse fly (*G. morsitans*) [13]. These symbionts could be found either dispersed throughout host body (e.g., in haemocoel) or association with bacteriomes where the p-symbionts are present. Some biological impacts of these endosymbionts have been observed, with a variety of negative and positive impacts on host biology. For instances, they can manipulate host reproduction, become a parasitic agent reducing host fitness and service the host biology as beneficial symbionts, for instance providing protection.

1.1.2 Symbionts that manipulate host reproduction

The transmission of heritable microbes is generally restricted to the maternal lineage (uniparental inheritance). The lack of transmission through male hosts may select for symbiont traits that favour the production and survival of female hosts. This action commonly only benefits the symbionts, by increasing the chance to transmit. Selection favours strains that cause their host to produce high numbers of females, through 'sex-ratio distortions'. Sex-biased manipulative mechanisms can be induced through several processes, i.e., feminisation, parthenogenesis induction and male-killing phenotype (see examples in Table 1.1). As has already been said, the imbalance of sex is commonly costly to the host. In male-killing mechanism, 50% of a progeny brood (the sons) die. This means the female mothers have also lost 50% of their reproductive energy to produce the dead males. In parthenogenesis induction, the embryos are formed without sexual recombination, which makes the lineage lose variation and likely to become extinct in the medium term, due to the accumulation of deleterious mutations (Muller's ratchet) [14, 15].

Table 1.1 Incidence of reproductive parasitism (RP) in invertebrate hosts.

Endosymbionts	Host		References
	Order	Species	
♦ **Feminization**			
Wolbachia	**Class: Insecta**		
	Hemiptera	*Zyginidia pullula*	[16]
	Lepidoptera	*Eurema hecabe*	[17, 18]
		E. mandarina	[19, 20]
	Class: Malacostaca		
	Isopoda	*Armadillidium vulgare*	[21]
		Cylisticus convexus	[22]
		Sphaeroma rugicauda	[23]
Cardinium	**Class: Insecta**		
	Hymenoptera	*Encarsia hispida*	[24]
	Class: Arachnida		
	Trombidiformes	*Brevipalpus californicus*	[25]
		B. phoenicis	[26]
Microsporidia	**Class: Malacostraca**		
	Amphipoda	*Gammarus duebeni*	[27]
♦ **Male killing**			
Wolbachia	**Class: Insecta**		
	Coleoptera	*Adalia bipunctata*	[28]
		Coccinella undecimpunctata	[29]
		Tribolium madens	[30]
	Diptera	*Drosophila bifasciata*	[31, 32]
		D. borealis	[33]
		D. innubila	[34]
	Lepidoptera	*Acraea encedon*	[28, 35]
		A. encedana	[36]
		Hypolimnas bolina	[37]
		Ostrinia furnacalis	[38]
		O. orientialis	[39]
		O. scapulalis	[40]
		O. zaguliaevi	[39]
	Class: Arachnida		
	Araneae	*Oedothorax gibbosus*	[41]
	Pseudoscorpionida	*Cordylochernes scorpioides*	[42]
Spiroplasma	**Class: Insecta**		
	Neuroptera	*Mallada desjardinsi*	[43]
	Coleoptera	*Adalia bipunctata*	[44]

		Anisosticta novemdecimpunctata	[45]
		Harmonia axyridis	[46, 47]
	Hemiptera	*Acyrthosiphon pisum*	[48, 49]
	Lepidoptera	*Danaus chrysippus*	[50]
		Ostrinia zaguliaevi	[51]
	Diptera	*Drosophila nebulosa*	[52]
		D. neocardini	[53]
		D. melanogaster	[54, 55]
		D. ornatifrons	[53]
		D. paraguayensis	[53]
		D. wilistoni	[56]
Rickettsia	**Class: Insecta**		
	Coleoptera	*Adalia bipunctata*	[44, 57, 58]
		A. decempunctata	[44]
		Brachys tessellatus	[59]
		Propylea japonica	[60]
Arsenophonus	**Class: Insecta**		
	Hymenoptera	*Nasonia vitripennis*	[61]
Flavobacteria	**Class: Insecta**		
	Coleoptera	*Adonia variegata*	[62]
		Coccinula sinensis	[63]
		Colemegilla maculata	[64]
Microsporidia	**Class: Insecta**		
	Diptera	*Anopheles quadimaculatus*	[65, 66]
Hamiltonella	**Class: Insecta**		
	Coleoptera	*Chilomenes sexmaculata*	[67]
♦ **Parthenogenesis**			
Wolbachia	**Class: Insecta**		
	Hymenoptera	*Aphytis diaapidis*	[68, 69]
		A. lingnanensis	[68, 70]
		Aponanagyrus diversicornis	[71]
		Asobaara japonica	[72]
		Diploepsis rosae	[73]
		Encarsia formosa	[74]
		Eretmocerus mundus	[75]
		Gronotoma micromorpha	[76]
		Muscidifurax uniraptor	[77, 78]
		Leptopilina clavipes	[79]
		Telenomus nawai	[80]
		Trichogramma brevicapillium	[81]
		T. chilonis	[82]

		T. cacoeciae	[83]
		T. cordubensis	[81, 82]
		T. deion	[81, 82]
		T. embryophagum	[81, 82]
		T. evanescens	[81, 82]
		T. kaykai	[84]
		T. oleae	[82]
		T. platneri	[81, 82]
		T. pretiosum	[81, 82]
	Thysanoptera	*Franklinothrips vespiformis*	[85]
	Class: Arachnida		
	Trombidiformes	*Bryobia* sp.	[86]
		B. praetiosa	[86]
Cardinium	**Class: Insecta**		
	Hymenoptera	*Encarsia berlesei*	[87]
		E. critina	[87]
		E. hispida	[87]
		E. pergandiella	[87]
		E. protransvena	[87]
Rickettsia	**Class: Insecta**		
	Hymenoptera	*Neochrysocharis formosa*	[88, 89]
		Pnigalio soemius	[90]

♦ **Cytoplasmic Incompatibility**

Wolbachia	**Class: Insecta**		
	Hemiptera	*Laodelphax striaellus*	[91]
		Orius strigicollis	[92]
	Diptera	*Ades albopictus*	[93-95]
		Bactrocera oleae	[96]
		Ceratitis capitata	[97]
		Culex pipiens	[98-101]
		Drosophila melanogaster	[55, 102]
		D. simulans	[102, 103]
		D. pseudotakahashii	[104]
	Lepidoptera	*Eurema hecabe*	[105]
		Colias erate poliographus	[106]
	Hymenoptera	*Cardiocondyla obscurior*	[107]
		Habrobracon hebetor	[108]
		Nasonia giraulti	[109]
		N. longicornis	[109]
		N. vitripennis	[109]
		Spalangia endius	[110]

Cardinium	**Class: Insecta**		
	Hymenoptera	*Encarsia pergandiella*	[111]
		E. suzannae	[112]
Rickettsiella	**Class: Arachnida**		
	Araneae	*Mermessus fradeorum*	[113]

1.1.3 Cytoplasmic incompatibility (CI)

Reproductive parasitic activity does not solely involve sex ratio distortion but can also be expressed in the phenotype of CI, in which there is embryonic death when paternal hosts carry the symbiont infection but maternal hosts do not. The mechanisms behind this trait are varied. The incompatible modification can occur at a pre-fertilization step during a male spermatogenesis [114] to the level of post-fertilization during embryogenesis [115, 116]. There are two basic types of CI based on crossing relationships. Unidirectional CI involves only one symbiont strain. It occurs when an infected male mates with an uninfected female, but it is rescued when the female is infected with the same symbiont strain as male. Bidirectional CI involves two symbiont strains. In this case incompatibility is observed when males and females are infected with different symbiont strains. The mating between incompatible strains lead to the offspring death (Figure 1.1).

The phenomenon of CI selects against uninfected lineages, which become sterilized in incompatible matings. As the infected females can mate either with infected or non-infected males and produce viable offspring, infected lineages are selected for (see more examples in Table 1.1).

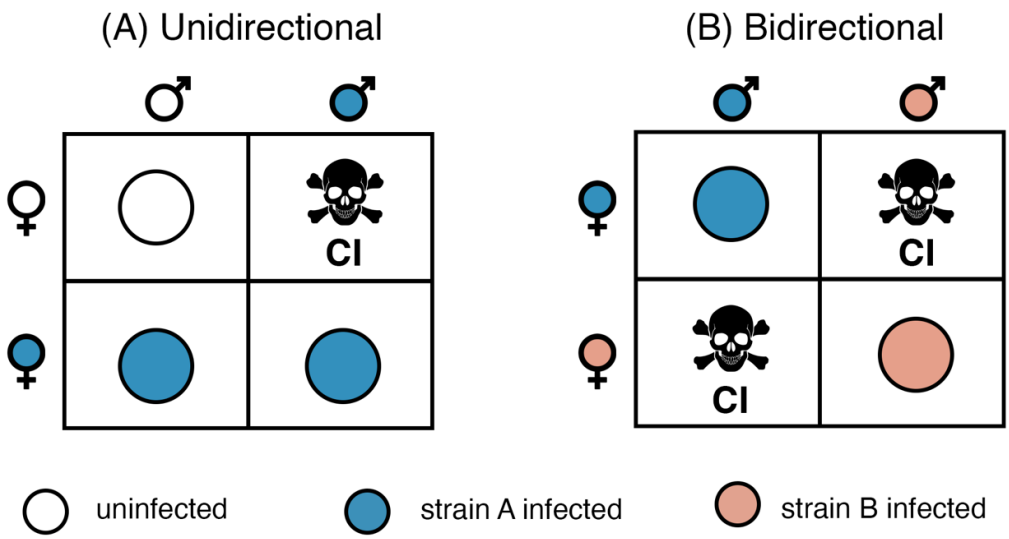

Figure 1.1 Two basic types of cytoplasmic incompatibility. (A) Unidirectional CI occurs when infected male mates with uninfected female. All other cases are compatible to form viable offspring. (B) Bidirectional CI occurs when mating males and females are infected with different strain of endosymbionts.

1.1.4 Fitness impacts of symbiont association

Despite the interspecies interaction between symbionts and hosts falling along the parasitism-mutualism continuum, the symbiont always benefits from the host as a vehicle for transmission; whether the host benefits however varies. Harbouring endosymbionts by default will always lead to a fitness cost to the host because of the additional energy need to keep the bacteria alive. However, this cost can be balanced by the symbiont conferring fitness benefit (see example in Table 1.2).

Commonly, the impact of symbionts on host fitness is established by comparing the biology of hosts with and without symbiont association [1, 72, 117, 118]. This process is sometimes challenging as many study systems are hard to maintain in laboratory conditions and some host species have multiple symbiotic partners whose individual influences may be hard to dissociate. Antibiotic treatments have been used for curing a symbiont to create non-infected host lines [1, 12, 72, 118-120], but these can be a problematic because it is difficult to distinguish between the effect of the antibiotic or the effect of losing the symbiont [121]. Further, they may be impossible to use in the presence of an obligatory association. However, recent studies can also predict the roles of symbionts from an investigation of symbiont genomes and their predicted capacities [2, 122, 123]. Nevertheless, the role of symbionts remains poorly understood in many cases.

Table 1.2 Examples of biological impacts of endosymbiont on invertebrate hosts

Symbionts	Host	Biological fitness	Ref.
♦ **Benefits**			
Wolbachia	**Class: Insecta**		
	Order: Diptera		
	Drosophila melanogaster	Protection against RNA virus	[124-126]
		Increase fecundity in the flies from temperate regions, but this effect is opposite to the flies from tropical regions	[127]
	Order: Hemiptera		
	Cimex lectularius	Supplement essential B-vitamin	[6]
	Order: Hymenoptera		
	Asobara tabida	Necessary for oogenesis	[128]
Wigglesworthia	**Class: Insecta**		
	Order: Diptera		
	Glossina sp.	Supplement essential B-vitamin	[129]
Blattabacterium	**Class: Insecta**		
	Order: Blattarea		
	Periplaneta americana	Provisional role on supplementing essential nutrition	[122]
Arsenophonus	**Class: Insecta**		
	Order: Hemiptera		
	Nilapavata lugens	Pesticide resistance	[130]
Buchnera	**Class: Insecta**		
	Order: Hemiptera		
	Acythosiphon pisum	Supplement essential amino acids	[131, 132]
	Sitobion avenae	Supplement essential nutrition on wing dimorphism	[118]
Regiella	**Class: Insecta**		
	Order: Hemiptera		
	Acythosiphon pisum	Protection against fungus	[133, 134]
Rickettsia	**Class: Clitellata**		
	Subclass: Hirudinea		
	Torix targoi	Potentially increase body size	[135]
	Hemiclepsis marginata	Potentially increase body size	[135]

	Class: Arachnida		
	Order: Ixodida		
	Ixodes pacificus	Provisional role in folate synthesis for the tick	[123]
	Class: Insecta		
	Order: Psocoptera		
	Liposcelis bostrychophila	Obligate to oogenesis	[10]
	Order: Hemiptera		
	Bemesia tabaci	Thermotolerance	[136, 137]
Streptomyces	**Class: Insecta**		
	Order: Hymenoptera		
	Philanthus triangulum	Protection against fungus	[138]
Spiroplasma	**Class: Insecta**		
	Order: Diptera		
	Drosophila melanogaster	Protection against a parasitoid wasp	[139]
	D. neotestacea	Protection against nematodes	[140]
Cardinium	**Class: Insecta**		
	Order: Hemiptera		
	Encarsia inaron	Increase male reproduction and survival	[141]
♦ **Costs**			
Wolbachia	**Class: Insecta**		
	Order: Hemiptera		
	Encarsia inaron	Reduce fecundity	[141]
Hamiltonella defensa	**Class: Insecta**		
	Order: Hemiptera		
	Rhopalosiphum padi	Reduce feeding efficiency of insect	[142]
Rickettsia	**Class: Arachnida**		
	Order: Araneae		
	Erigone atra	Limits host dispersion, potentially reduce gene flow	[143]
	Class: Insecta		
	Order: Hymenoptera		
	Spalangia endius	Reduce fecundity	[110]
Cardinium	**Class: Insecta**		
	Order: Hemiptera		
	Encarsia inaron	Reduce fecundity	[141]
Spiroplasma	**Class: Insecta**		
	Order: Hemiptera		
	Laodelphax striaellus	Reduce fertility	[91]

1.2 *Rickettsia*

Rickettsia is a genus in the order Rickettsiales within the alpha-proteobacteria. All bacteria in this genus are gram-negative, nonspore-forming and strictly intracellular symbionts of eukaryotic host cells [144, 145]. The bacterial genus '*Rickettsia*' was named after a superb pathologist, Howard Taylor Ricketts (1871-1910), who pioneered studies of Rocky Mountain spotted fever [146, 147], a deadly rickettsiosis disease. During and immediately after the World War I, the outbreak of epidemic typhus rickettsiosis caused millions of deaths. Since then, *Rickettsia* have been recognised as arthropod-borne diseases that can significantly impact humankind, and which were untreatable before the development of antibiotics.

1.2.1 *Rickettsia* in the aspects of arthropod borne disease

Historically, *Rickettsia* were classified into three major groups according to serological characteristics from infected patients: typhus, spotted-fever and scrub-typhus groups [148]. With a new genomic classification, based on the sequence of fifteen proteins, they were divided into four major groups, i.e., spotted-fever, typhus, transitional and ancestral group (Figure 1.2) [149]. All of these pathogenic *Rickettsia* use blood-feeding arthropods, e.g., ticks, mites, louse and fleas, as the vectors for their transmission. Following the arthropods bite (or a case that they leave infected faeces on the scratched skin), *Rickettsia* disseminate into the blood and infect the vascular endothelium of skin and many internal organs (e.g., brain, lungs, heart and kidneys) [145]. They invade into the host cells by phagocytosis, the same entry mode used in arthropod host cells [145]. Common symptoms in people infected with *Rickettsia* include high fever, rash and cutaneous necrosis [145]. In 1994, *Rickettsia* was found in association with insects as an endosymbiont [57]. This strain, which was a male-killing heritable microbe from ladybird beetles, was phylogenetically ancestral to all known clades at the time and did not transmit to vertebrates. A number of related strains have been observed in recent times, and it is now recognised that *Rickettsia* are a common invertebrate symbiont [144, 150].

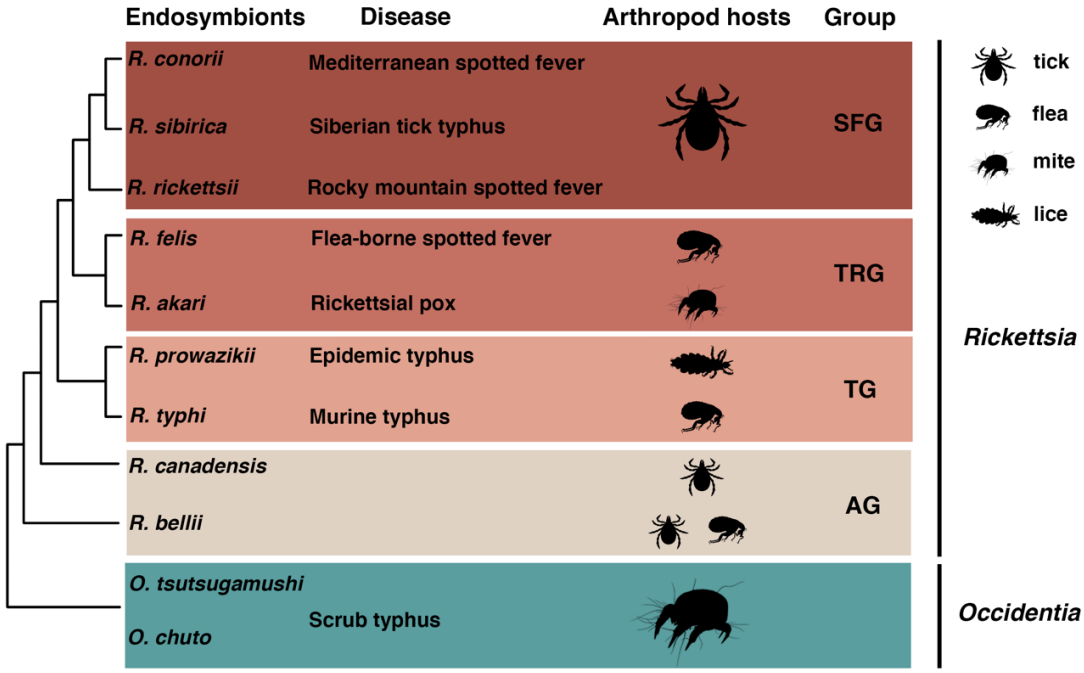

Figure 1.2 Phylogeny of pathogenic *Rickettsia.* The inferred topology is estimated under parsimony of fifteen *R. felis* proteins involved in metabolic pathways, obtained from the study of Gillespie *et al*. [151]. All the analysed *Rickettsia* spp. are classified into four group, i.e., spotted fever (SFG), transition (TRG), typhus (TG) and ancestral (AG). The middle columns are diseases in the accidental human host, the hosts and arthropod vectors of each *Rickettsia* spp. Image adapted from Fuxelius *et al*. [149].

1.2.2 Arthropod-associated *Rickettsia*

The vast majority of *Rickettsia* strains live as endosymbionts in arthropods and are not vertebrate pathogens. In contrast, they have been observed to play important roles in arthropod ecology, evolution and behaviour. The first incidence of this strain discovered in a ladybird (*Adalia bipunctata*) by the observation of Werren *et al*. in 1994 [57]. This strain can manipulate reproduction in the beetle host by inducing a male-killing phenotype in infected females. *Rickettsia* have been highlighted as one group of the endosymbiotic bacteria that impacts on insect evolution. Like other endosymbionts, *Rickettsia* can be observed in a wide range of eukaryotic species [144, 150] and can be transmitted through host generations vertically or through occasional inter species transfers [152].

1.2.3 Transmission patterns and impact on host biology

In the field of epidemiology, horizontal transmission of *Rickettsia* seems to be the major route of disease spread [153]. However, the inheritance of this vertebrate pathogen via transstadial and vertical transmission seems to be a factor that maintains the symbionts in the arthropod vectors [150]. In most cases, *Rickettsia* are obligate or facultative endosymbionts of their invertebrate hosts, which can be in the form of parasitic or mutualistic relationships.

Whilst vertical transmission dominates arthropod-*Rickettsia* symbiosis, interactions may also be established when the invertebrate hosts acquire the symbionts from environment or their diet. For instance, the sweet potato whiteflies (*Bemisia tabaci*) can receive *Rickettsia* from the consumption of infected plants [152, 154]. In this case, *Rickettsia* and the insect hosts form a facultative mutualistic relationship in which the hosts provide shelter and symbionts provide protection from other pathogenic bacteria (see table 1.2) [155]. This mixed transmission mode (vertical and horizontal) is also observed in the case of plant pathogenic *Rickettsia* (papaya bunchy top disease) which has a leaf hopper (*Empoasca papayae*) as a vector [156].

Most invertebrate-associated *Rickettsia* are acquired strictly through the maternal line, which indicates an occurrence of co-evolution of *Rickettsia* with their

host. In these cases, *Rickettsia* are likely to have influences on host survival and reproduction. The influences can be expressed across the parasitism-mutualism continuum. For example, in the pea aphid (*Acyrthosiphon pisum*), vertically inherited *Rickettsia* improve the insect's resistance against *Pandora*, a pathogenic fungus [157], while related strains help to increase fecundity [158], thermotolerance [136] stimulate oogenesis [10] and increase body size [135]. On the other hand, *Rickettsia* can form a parasitic relationship with their host. For example, *Rickettsia* can alter biological fitness (e.g., slowing host development [110], see Table 1.2) or develop into a reproductive parasitism (e.g., male-killing phenotype [57] and parthenogenesis induction [90], see Table 1.1). Like other symbioses, the type of relationship and host impacts vary depending on the symbiont strains and host species.

1.2.4 Host range

Rickettsia were originally recognised as causing arthropod-borne diseases. Most hosts of the vertebrate pathogenic *Rickettsia* are blood-feeding arthropods, e.g., ticks (*Amblyomma* sp. and *Demacentor* sp.[159, 160]), human lice (*Pediculus hominis corporis* [161]) and fleas (*Xenopsylla cheopis* and *Ctenocephalides felis* [162, 163]). Nevertheless, recent intensive molecular studies have found *Rickettsia* in wide range of eukaryotic organisms. *Rickettsia*'s host spectrum ranges from Protista, i.e., amoeba (*Nuclearia pattersonii*) [164] to invertebrates in the Animalia Kingdom (e.g., leeches, spiders and insects [144]). Additionally, *Rickettsia* species have also been reported infection in plant as reservoir hosts [152, 156]. However, in this section I will focus on host groups that are invertebrates, which are better studied. At the time of commencing this thesis, the majority of *Rickettsia* host species were from the insect orders Hemiptera, Coleoptera, Diptera and Hymenoptera. Other insect orders have been observed as hosts, but at lower frequency [144].

Although the range of hemipteran hosts known to carry *Rickettsia* is taxonomically narrow, the insects in this group that host *Rickettsia* are mainly phloem-feeding insects which have an impact in agricultural and pest management fields. Previous reports were related to the discovery of plant pathogenic *Rickettsia* in the past (papaya leafhopper host) [156]. A closely related *Rickettsia* of those were

also found in aphids (*Acyrthosiphon pisum*) [12, 165] and whiteflies (*Bemisia tobachi*) [166] and distantly related strains were observed in leafhoppers (e.g., *Nephotettix cincticeps* [11], *Macrosteles striifrons* and *M. sexnotatus* [167]). Hymenopterans have also been reported as host species in several parasitoid and gall wasps, e.g., family Eulophidae [90, 168]

Coleopterans and dipterans that are hosts for *Rickettsia* have a much wider species range. *Rickettsia* have been reported within the insect spectrum that comprise the species that live or are associated with freshwater and terrestrial ecosystems. A few water beetles in the family Dytiscidae (*Deronectes* sp.) were reported in association with *Rickettsia* strains that were abundant in other freshwater species [169]. Other families that live on land (e.g., Curculionidae [170-172] and Coccinellidae [57, 173]) have been widely reported with other *Rickettsia* strains. The Diptera are another hot-spot for *Rickettsia* symbionts. Many species of dipteran are human ectoparasites, e.g., Culicidae (mosquitoes) [174], Ceratopogonidae (biting midges) [175, 176], Glossinidae (tsetse fly) [177], and Psychodidae (sand fly) [178]. In addition, some of these are non-haematophagous dipterans, e.g., Dolichopodidae and Empididae (long-leg fly) [179]. Apart from these insect orders, there are a variety of other insect groups which have been reported for hosting non-vertebrate pathogenic *Rickettsia*, e.g., Collembola [180], Psocoptera [10], Neuroptera [181], Lepidoptera [182] and Siphonaptera [183].

A particular hot-spot for *Rickettsia* presence is the Arachnida. *Rickettsia* have been observed in the Ixodidae [184], Acaridae [185], and Araneae [186]. The first two orders commonly harbour vertebrate pathogenic *Rickettsia* while the last is a hot-spot for non-pathogenic strains to vertebrates [186-189]. Freshwater species, e.g., Amphipoda [190] and non-arthropod invertebrates (i.e., Hirudinea in Annelida) [9, 135] have also been observed to carry *Rickettsia*.

1.2.5 Diversity of *Rickettsia*

Our understanding of the genetic diversity of *Rickettsia* has expanded, first since the development of PCR, and more recently next-generation sequencing. In the past, *Rickettsia* were identified into species levels by the identification generally

based on *16S rRNA* sequence with the assumption that sequences of >97% identity represent the same species [191]. However, it is more complex and difficult to classify endosymbiont *Rickettsia* into species level or designate a new species name, due to the fact that some strains have broad variations when they live in different hosts [150, 175, 181]. Another simple approach for endosymbiont classifications is likely to identify the strain according to host species rather than define the strain with the specific epithet [150, 175, 181].

To date, *Rickettsia* have been classified into 9 major groups based on studies of Weinert *et al*. 2009 [182], Weinert 2015 [144] and Castelli *et al*. 2016 [192], with the exclusion of Hydra group [144, 182], which is now considered as *Candidatus* Megaira (a *Rickettsia* sister genus) [192]. Vertebrate pathogenic strains are affiliated in transitional, typhus, spotted fever, canadensis and bellii groups (Figure 1.3). Strains lacking a vertebrate infection phase were found in the other groups, as well as the bellii group (shared with pathogens). The bellii clade represent a diverse assemblage of strains from arthropod hosts, while the rhyzobius, meloidae and adalia clades currently have a narrower spectrum of recognised host organisms. It is now clear the torix clade comprises the largest diversity of host organisms, from amoebae through leeches, to an array of arthropods (amphipods, spiders and insects, Figure 1.3).

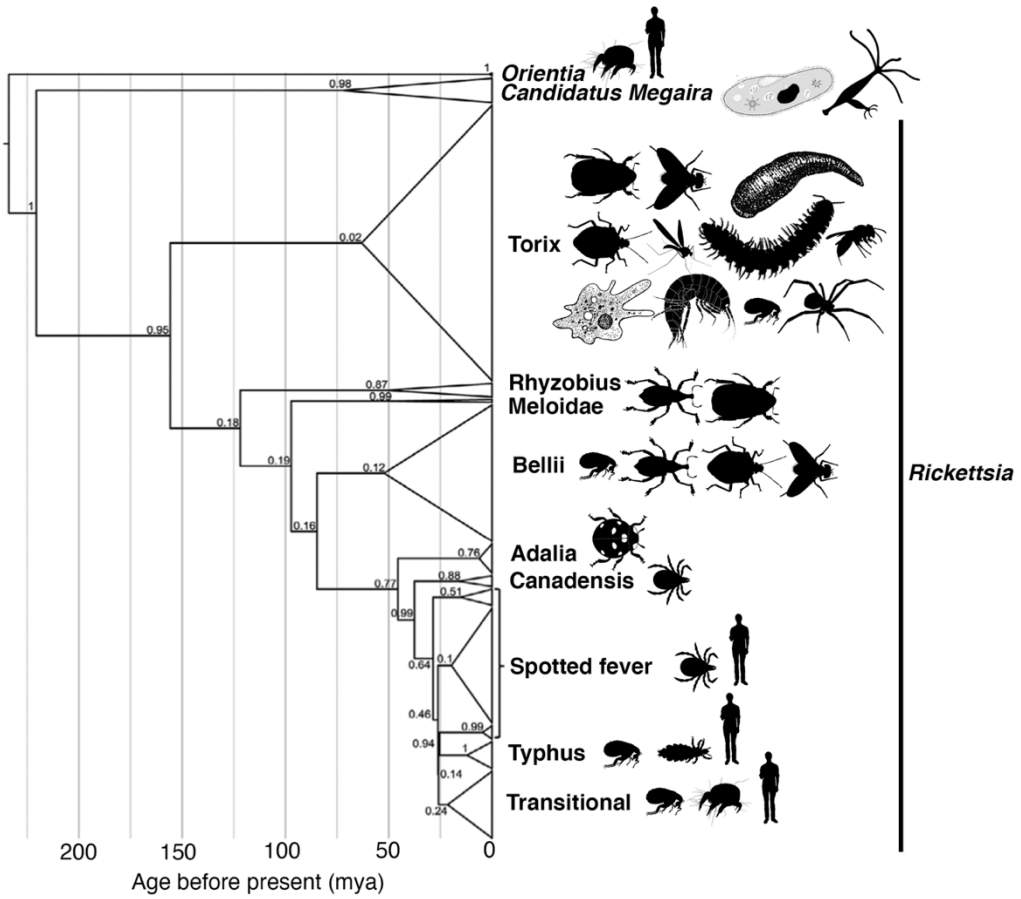

Figure 1.3 Phylogeny of *Rickettsia* based on *16S rRNA* and *gltA* gene with molecular clock dating. Topologies indicate relationship among *Rickettsia* lineages. On the right-side showing illustrations of representative host groups. Bootstrap support is given on the node. The image is adapted from Weinert *et al*. [144] and Castelli *et al*. [192].

1.3 Torix group *Rickettsia*

The increased number of *Rickettsia* studies reporting the presence of torix *Rickettsia* has produced a wider appreciation of this group of bacteria and the invertebrates that harbour them [150]. Whilst the number of recorded symbioses involving torix group strains are growing, our understanding of host preferences and biological impacts of the symbionts is poorly established [9, 169, 175, 182].

1.3.1 Incidence of torix group *Rickettsia*

Torix group *Rickettsia* were originally described in two freshwater Japanese leech species, i.e., *Torix targo*i and *Hemicrepsis marginata* in the study of Kikuchi *et al*. [9]. Both host species are members of family Glossiphoniidae. *Rickettsia* localization was observed in epidermal cells, and in the oesophagus and intestine of *T. tagoi*. The freshly hatched offspring of this leech species all carried *Rickettsia* infection suggesting the symbionts are inherited via vertical transmission. A later study in 2005 [135] found another *Torix* leech species (*T. tukubana)* hosted *Rickettsia.* This study also revealed the potential roles of torix *Rickettsia* in enlarging body-size of the three leech species.

1.3.2 Torix *Rickettsia* hosts

Currently, hosts of torix *Rickettsia* are mainly organisms from freshwater ecosystems, e.g., protists [164], hirudineans [9, 135], crustaceans [190, 193] and other freshwater associated insects [169, 175, 182]. On the other hand, some hosts are terrestrial, e.g., diplopods [194], arachnids [186] and terrestrial insects [155, 165, 195, 196], although these are considered to be in a minority. This has led to the 'freshwater hot-spot' hypothesis [182], that torix *Rickettsia* might be more abundant in freshwater than terrestrial communities. However, there are some freshwater invertebrates that potentially harbour torix *Rickettsia* where there has been no record (e.g., insects of the order Odonata). Additionally, this hypothesis has not yet been directly tested, through an unbiased screen completed comparing freshwater and terrestrial communities.

1.4 General objectives, study system and directions for this thesis

Torix *Rickettsia* are likely abundant in the freshwater biome, but our knowledge of this group remains poor. The odonates represent a freshwater insect group that is diverse and well known but has yet to be observed as a host of torix *Rickettsia*. Odonates are commonly used as an ecological model, understanding symbioses in this group is important. Further, the freshwater hot-spot hypothesis has yet to be formally tested: it has emerged from a review of the records that have been published, rather than a hypothesis driven study. Finally, the biological impact of torix *Rickettsia* in their host species are very poorly understood, and there is a need to establish a model study system. These lead to the objectives and hypotheses of this thesis.

1.4.1 Chapter 2: Diversity of *Rickettsia* in order Odonata

To explore incidence and prevalence of torix *Rickettsia* in insects in the order Odonata (dragonflies and damselflies), I investigate the presence of torix *Rickettsia* infection using a PCR assay screen of diverse odonate species from South America and Europe. These data are combined with the sequence of marker genes to establish the diversity of infections. For one case study, *Coenagrion puella*, I visualise the symbiont within the host using fluorescence in situ hybridisation (FISH) to establish the likelihood of heritable symbiosis. Finally, a case study is developed indicating *Rickettsia* may drive the lack of a mtDNA barcoding gap between two species, *C. puella* and *C. pulchellum*.

1.4.2 Chapter 3: Screening of freshwater and terrestrial arthropod taxa

In this chapter, the hypothesis of 'freshwater hot-spot' is tested through a screening approach, comparing aquatic and terrestrial species. This study will utilize both newly collected material and historically curated DNA template from a previous study to allow the incidence of *Rickettsia* in these two biomes to be established. The approach is based on PCR assays, combined with Sanger sequencing of products to confirm phylogenetic affiliation of the strains discovered.

1.4.3 Chapter 4: Incidence of torix *Rickettsia* in the common bed bug

A key deficit in our understanding of torix *Rickettsia* symbiosis are the biological impacts on their host. This deficit is caused by a lack of a laboratory model for analysis. In Chapter 3, I revealed the presence of torix *Rickettsia* infection in the common bed bug (*Cimex lectularius*) from curated DNA templates collected from the laboratory colony in University of Sheffield. In this chapter, I establish the utility of a bed bug-*Rickettsia* system to explore the distribution of torix *Rickettsia* across populations and in individual tissues. I screened through various laboratory bed bug populations that were originally collected from Africa, Mainland Europe and Great Britain with PCR assays alongside with a screening of cimicid allies. The FISH approach was used to localise the symbiont infection in bed bug tissue. Male and female *C. lectularius* from infected and uninfected lines were allowed to mate for observing the transmission passage in the offspring.

1.4.4 Chapter 5: Torix *Rickettsia*-Bed bug interaction

The previous chapter established the presence of *Rickettsia* infected and uninfected lineages of bedbugs that were otherwise isogenic. This affords the capacity to determine the biological influences of *Rickettsia*, which would otherwise be impossible (because antibiotic treatment would disrupt the obligate symbiosis with *Wolbachia*). I investigated a few biological impacts of the symbiont on bed bugs. Impacts on host life history and fecundity were compared between the two lines (i.e., development time, fecundity, body-size and longevity) and evidence of reproductive parasitism assessed (i.e., sex-ratio distortion and CI).

The thesis ends with a discussion, which summarises the findings of the thesis, and details key knowledge gaps for onward study.

CHAPTER 2

Incidence and diversity of torix *Rickettsia* – Odonata symbioses

Publication and author contributions

All data in this chapter is published in *Microbial Ecology* as Thongprem *et al.* 2020 [197]. The odonate materials in the Broad-screen were contributed by Prof David Thompson, University of Liverpool, and Dr M. Olalla Lorenzo-Carballa, Universidade de Vigo, Spain. DNA extraction, PCR screening, Sanger sequencing and Table analysis were performed by me with the help of Helen Davison, University of Liverpool. MLST, phylogenetic and FISH analysis were performed by me.

2.1 ABSTRACT

Heritable microbes are an important component of invertebrate biology, acting both as beneficial symbionts and reproductive parasites. Whilst most previous research has focussed on the '*Wolbachia* pandemic', recent work has emphasised the importance of other microbial symbionts. In this study, I present a survey of odonates (dragonflies and damselflies) for torix group *Rickettsia*, following previous research indicating that this clade is common in other aquatic insect groups. PCR assays were used to screen for a broad range of odonates from two continents and revealed 8 of 75 species tested were infected with *Rickettsia*. I then conducted further deeper screening of UK representatives of the Coenagrionidae damselfly family, revealing 6 of 8 UK coenagrionid species to be positive for torix *Rickettsia*. Analysis of *Rickettsia* gene sequences supported multiple establishments of symbiosis in the group. Some strains were shared between UK coenagrionid species that shared mtDNA barcodes, indicating a likely route for mitochondrial introgression between sister species. There was also evidence of coinfecting *Rickettsia* strains in two species. FISH analysis indicated *Rickettsia* were observed in the ovarioles, consistent with heritable symbiosis. In conclusion, torix *Rickettsia* represent an important associate of odonates, being found in a broad range of species from both Europe and South America. There is evidence that coinfection can occur, vertical transmission is likely, and that symbiont movement following hybridization may underpin the lack of 'barcoding gap' between well-established species pairs in the genus. Future work should establish the biological significance of the symbioses observed.

2.2 INTRODUCTION

2.2.1 General introduction to Odonata

Insects in the order Odonata are cosmopolitan and highly diverse. There are 6,298 species around the world have been described to date [198]. The insects in this order are called 'odonates' for the comprehensive term. They can be classified into three suborders. The smallest and mostly neglected suborder is Epiophlebioptera [199], which consists of only three species and includes the member of the extinct suborder Anisozygoptera [200]. The other two main suborders are well known, i.e., suborder Zygoptera or 'damselflies' and Anisoptera or 'dragonflies.' Dragonflies and damselflies are ecologically important taxa especially for conservation and citizen sciences as they can be easily identified and used as indicators for monitoring biodiversity and the health of freshwater habitat [201, 202].

Like other hemimetabolous insects, odonate larvae molt into adults without pupal eclosion. The aquatic larvae need 5-14 molts before metamorphosis to flying adults in terrestrial habitats [203-205]. Odonates are associated with freshwater ecosystem over their whole lifecycle. The larvae live as predators under the water, feeding on small fishes and aquatic invertebrates [203]. Because of the wide variation in breeding seasons, the durations of the odonate life cycle are varied, ranging from multivoltine, a group of odonate species that can produce few generations within one year, to partivoltine, species that need more than two years to complete one generation [206]. When metamorphosis has begun, the final naiad stadium needs to switch from a gill-breathing to an air-breathing insect. They need to migrate onto vegetation or other objects above the water. When the adult form emerges from the exuviae, the abdomen is elongated, and the wings that are generated from the wing buds of naiad thoracic dorsum are expanded [206]. The structure of thoracic muscles that connect straight to their wings possess a special wing movement in odonates. This movement is effective enough to support the powerful flight ability which benefits them to live in the terrestrial habitats as aerial predators. Most adults are generalist predators feeding on small insects, e.g., order Diptera (small flies, mosquitoes and midges), Coleoptera (small beetles), Hemiptera (plant hoppers and

small bugs), Lepidoptera (small butterflies) and Hymenoptera (honey bees and ants) [207]. Apart from this, the wings also support them in long-distance over continental migrations, which are successful in many dragonfly species [208, 209].

2.2.2 Dispersion across geographical area

One of the most marked 'achievements' of the insects in this order are migrations. Many dragonfly species can be found across continental regions; for examples, the common green darner (*Anax junius*) and the wondering glider (*Pantala flavescens*) [208, 209]. The latter species is common in almost all continents; and holds the record of the longest-distance migratory insect of the world [209]. This species can be observed in far distanced tectonic plates, e.g., north-eastern America [210], north-eastern China [211], over the Baltic sea [212] and Amsterdam Island in the South Indian ocean [213]. Damselflies are contrary, according to the small size of their wing spans, making damselfly mobility less effective than that of dragonflies when they are dispersing toward geographical barriers, especially the habitats that are isolated from the main continental regions like islands. The geographical formation of islands enhances allopatric speciation for many organisms that have become limited in their distributions once they have been introduced onto an island [214]. With the influence of the geographical barrier, some damselfly species become endemic on particular islands [215] or even develop a unique phenotype, like asexual reproduction [216, 217].

The North American damselfly, *Ischnura hastata*, has been recorded as the only species of Odonata that has developed the phenotype of thelytokous parthenogenesis in populations that live on the Azores Islands [217]. A previous study has observed driving factors for this phenotypic adaptation and found that the parthenogenesis in this species was influenced by an apomictic mechanism when the damselflies speciated in the islands [216]. There was no evidence of endosymbionts involvement in this phenotype [216], unlike in cases of parthenogenesis in other invertebrates [72, 86, 87, 90]. However, some damselfly species have a wide distribution range, especially the areas where large tectonic plates are close or connected. For example, the azure damselfly (*Coenagrion puella*), a member of family

Coenagrionidae, have wide distribution in Europe. They are commonly distributed across Great Britain, mainland Europe and northern Africa, and classified by the IUCN as having Least Concern (LC) status at the global assessment scale [218]. Nevertheless, at the edge populations of northern Africa, e.g., Morocco and Algeria, the number of the populations are scarce [219], which resulted in unique genotypic variations [220] due to founder effects from the disconnect of this tectonic plate from mainland Europe. Moreover, the limited distribution of damselflies when they encounter geographical barriers, may this potentially be involved as a driving factor in the speciation of Zygoptera, which make damselflies are more diverse than dragonflies [198].

2.2.3 Family Coenagrionidae in British Isles

One of the big zygopteran families is Coenagrionidae, sometime called 'a pond damselfly' as they are the major odonates that inhabit lentic ecosystems. The family consists of 1,353 species around the world [198]. There are 13 species distributed throughout Great Britain and Ireland, comprising the genera *Ceriagrion*, *Coenagrion, Enallagma, Erythromma, Ischnura* and *Pyrrhosoma* [221]. According to the colour of male adults, they are known as 'red and blue damselflies'. There are only a few common species widespread throughout the British Isles, e.g., Large red damselflies (*Pyrrhosoma nymphula*), Common blue damselflies (*Enallagma cyathigerum*) and Azure damselflies (*Coenagrion puella*). Indeed, almost half of the UK coenagrionid species are restricted residents, found in few locations. For instance, Small red damselflies (*Ceriagrion tenellum*) are found only in the southern part of Great Britain, and Irish damselflies (*Coenagrion lunulatum*) are found only in Ireland [221]. Most of the coenagrionid damselflies in the genus *Coenagrion* have restricted distributions and have become protected species, e.g., the Southern damselflies (*C. mercuriale*) and the Norfolk damselflies (*C. armatum*); the latter one is believed to be extinct from UK [221]. However, there are two common sister species of *Coenagrion* genus that disperse in many locations in British Isles, i.e., *C. puella* and *C. pulchellum.*

2.2.4 Mitochondrial DNA introgression of the two sister *Coenagrion* species

The azure damselflies (*C. puella*) and variable damselflies (*C. pulchellum*) are similar in their morphology. The adult male of *Coenagrion* damselflies are easily distinguished by comparing the shape of the black mark on 2nd abdominal segment [221] (Figure 2.1). Nevertheless, the mark of variable damselflies are diverse. Thus, the species are more difficult to identify when they are both found in the same location. *Coenagrion puella* are more abundant and have wider distribution than *C. pulchellum*. A decline in number of *C. pulchellum* has been observed. One of the hypotheses for this phenomenon is focused on hybridisation events when the two sister species are sympatric [222].

Hybridisation likely reflects negative consequences; a decrease in population size of a weaker species is likely, usually when the two interbreeding populations are unequal [223]. More strong genetic evidence in the two sister damselflies was provided in the study of Freeland and Conrad in 2002 [224]. They revealed the limited haplotypes variation of the two species in sympatric localities. One of the three haplotypes indicated the evidence of mtDNA introgression between the two species which supported the hybridisation hypothesis. However, the study of Lowe *et al*. in 2008 [225] showed more stronger genetic evidence using 12 microsatellite markers, which revealed the divergence of this sister species in the sympatric populations and concluded that 'they do not hybridise' [225]. It should be noted that, when the two species have introgressed identical mtDNA haplotypes, but they do not interbreed, this might be indicating the slow process of sympatric speciation [226, 227] or that the introgression is driven by a biotic factor, e.g., endosymbiont induction [228].

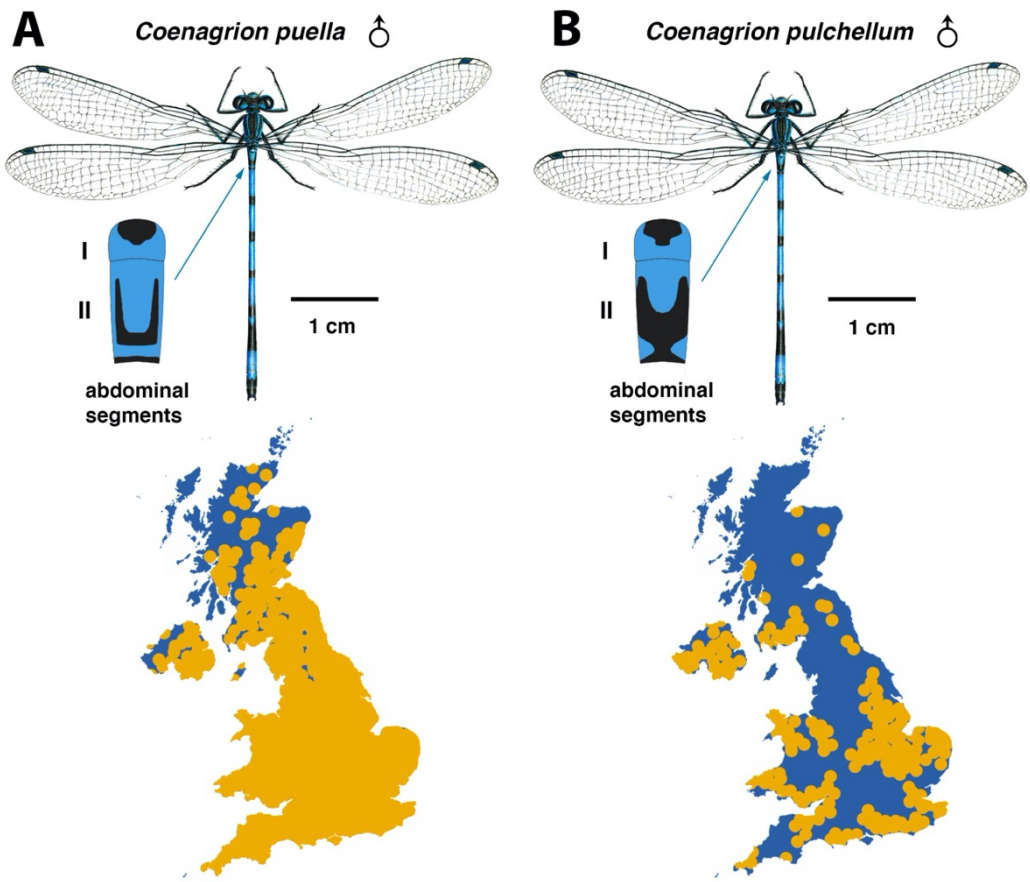

Figure 2.1 Illustrations of two sister *Coenagrion* species. the **A**: Azure damselfly (*C. puella*) and **B**: Variable damselfly (*C. pulchellum*) and their distribution in UK. The common markings on the dorsal back of 2nd abdominal segment, where the arrows are pointed. This mark of male adult is generally used as a morphological character to identify *Coenagrion* species. The UK distribution maps of the two species are modified from 'NBN Atlas occurrence download at http://nbnatlas.org. Accessed 23 September 2020'.

2.2.5 Symbionts in odonates

Odonates have recently been revealed as hosts for *Wolbachia* [229-231], the best-known heritable symbiont, which is estimated to infect over 50% of insect species [232]. In South-east Asia, 4 out of 33 screened odonate species harboured *Wolbachia* symbionts [230], while 16 odonates species from Indian populations were observed with high infection prevalence (~70%) [229]. For the South Pacific area, populations of *Nesobasis* and *Melanesobasis* damselflies that were distributed among the Fiji archipelago, were found to be a hot-spot for *Wolbachia* infection from an in-depth screen [231]. The study also investigated the biological impact of *Wolbachia* on the damselfly hosts, but there were no reproductive manipulations (e.g., feminization and parthenogenesis induction) observed in the system. However, the authors have suggested that *Wolbachia* might be one of the factors that drove the radiation of the two damselfly genera [231]. The commonness of this endosymbiont infection is seemingly moderate for this cosmopolitan insect. With the behaviour and ecology of odonates, they may potentially acquire other endosymbionts from the freshwater biomes and from their prey, but surveys for other members of the Rickettsiales have yet to be completed.

2.2.6 Torix *Rickettsia*, an endosymbiont in a freshwater biome

Whilst *Wolbachia* is not the only bacterial symbiont of insects, it is the best studied of terrestrial and, to a lesser extent, freshwater taxa [188]. The documentation for endosymbionts in freshwater insects is particularly poor when compared to terrestrial insects, with the notable exception of mosquitoes [233]. Recently, the presence of torix *Rickettsia* has been noted in a variety of aquatic invertebrate taxa. First discovered in *Torix* leeches [9, 135], hot-spots of torix *Rickettsia* have been observed in *Culicoides* biting midges [175], deronectid diving beetles [169] and dolichopodid flies [179]. To date, the impact of symbionts from this group on host biology is unclear, with the exception of bark lice (*Cerobasis guestfalica*), in which *Rickettsia* infection is associated with parthenogenetic reproduction by the host [10]. However, the symbiont infection is a potentially important aspect of biology that has generally been overlooked in aquatic insects.

2.2.7 Aims

Odonates are ecologically important species in freshwater habitats that also have potential to acquire *Rickettsia* bacteria from other members of the freshwater community. With their pronounced dispersal ability, they are likely to allow symbiont to hitchhike to other geographical areas. Investigating the incidence of *Rickettsia* in these cosmopolitan insects could help enrich biological and ecological knowledge of both symbiotic bacteria and odonate hosts. Exploratory research will hopefully encourage further studies in this aspect of insect-endosymbiont evolution.

To investigate the incidence of *Rickettsia* infection in odonates, I screened odonate samples with PCR assays. The screened species combined a broad sweep of biogeographical and taxonomic diversity. I also explored infection in-depth with a greater number of individuals in a damselfly family Coenagrionidae in the UK, which were readily available for collection. I performed FISH analysis of *Rickettsia* tropism in *Coenagrion puella* to establish if the symbiont is present in developing oocytes and thus determine the likelihood of vertical transmission.

2.3 METHODS

2.3.1 Sample collection and genomic DNA preparation

Existing odonate DNA and preserved leg material from previous studies [234-241] were sent to the laboratory in University of Liverpool to test the presence of *Rickettsia* (Table 2.1). These samples cover UK, South America, Mainland Europe and the Azores islands.

Several fresh specimens were collected in UK during 2016-2018. Sampling locations were observed only in UK, Cheshire, Merseyside and Hampshire (Table 2.1). Adult odonates were sampled using a butterfly net (12" diameter). The nymphs were collected from ponds that have aquatic weeds and from small streams with slow running water, using a pond dipping net (25x25 cm with 1 mm mesh size). The adults were identified directly in the field and then preserved in 100% EtOH, but the nymphs were preserved and brought back to the laboratory for identification.

All the preserved specimens (both nymphs and adults) were rinsed with fresh 100% EtOH. Legs from individual samples were cut with a sharp sterilised forceps and air dried in a room temperature. The leg materials were extracted for DNA using a Promega Wizard® Genomic DNA Purification kit (A1120, Promega, UK) adapted from the manufacture protocol. Briefly, the leg tissues were homogenised in 1.5 ml centrifuge tube with 150 µl of Nuclei Lysis solution. Non-relevant proteins were then precipitated by adding 50 µl of Protein Precipitation solution and kept the tube on ice for 5 min. The tube was centrifuged at 16,000 g for 4 min, and the supernatant containing the DNA was gently transferred into a new 1.5 ml centrifuge tube. DNA was precipitated by adding 200 µl of 100% Iso-propanol, which was mixed gently and centrifuged again at 16,000 g for 2 min. The supernatant was discarded, leaving a white DNA pellet in the tube. The pellet was washed with 200 µl of 70% EtOH twice, then centrifuged at 16,000 g for 2 min for each time. Finally, the supernatant was discarded, the DNA pellet was air dried and resuspended in 100 µl of molecular graded water. All DNA templates were stored at -20^{o}C.

The analysed material covered a total of 374 individuals from 80 species within 8 families, from the UK, South America, mainland Europe and the Azores (Table 2.1). The screen separated into two sections; 1) broad screen and 2) focussed UK coenagrionids screen. In the broad screen, 307 odonate samples covering 75 species were screened for *Rickettsia* infection to enable a global view of the infection prevalence (Table 2.1). After the detection of high infection frequency in two damselfly species in the family Coenagrionidae from the broad screen (in Results section), this generated an expansion of the focussed screen to include other UK coenagrionid damselflies. The Focussed screen observed 112 individuals of 8 UK damselfly species within the family Coenagrionidae (3 species from the broad screen; *Coenagrion puella, Enallagma cyathigerum* and *Ischnura elegans,* and 5 additional species; *Coenagrion pulchellum, C. mercuriale, Ceriagrion tenellum, Erythromma najas, and Pyrrhosoma nymphula*) to enable an in-depth view of the prevalence within species and any sex bias in presence (Table 2.1). The *Rickettsia* infections in the focussed screen were also subjected to further MLST analysis to observe their haplotype diversity in 5 house-keeping genes.

Table 2.1 Odonate samples. The broad screen includes 75 species covering South America, mainland Europe, the Azores and the UK. The focussed screen observed 8 coenagrionid species from the UK (3 species from broad screen and 5 species in addition), highlighted in bold. In Collection column, 'a' indicates the specimens that were freshly collected in this study, 'b' indicates the samples that were obtained from previous studies (see section 2.3.1). *N* indicates the total number of insects/species that passed the QC test in PCR assays (see section 2.3.2).

No.	Species	Family	Location	Collection	*N*	Adult	Nymph
Broad Global Screen							
	Suborder Anisoptera (Dragonflies)						
1	*Anax imperator*	Aeshnidae	Italy, Spain, Azores and continental Portugal	b	14	5	9
2	*Oxygastra curtisii*	Corduliidae	Tojal, Portugal	b	1	1	-
3	*Cannaphila vibex*	Libellulidae	Maquipucuna, Ecuador	b	1	1	-
4	*Erythrodiplax amazonica*	Libellulidae	Tiputini Ecuador	b	1	1	-
5	*E. kimminsi*	Libellulidae	Tiputini Ecuador	b	3	3	-
6	*E. unimaculata*	Libellulidae	Tiputini Ecuador	b	1	1	-
7	*Libellula depressa*	Libellulidae	Ness Gardens, Cheshire, UK	a	1	0	1
8	*Orthemis cultriformis*	Libellulidae	Tiputini, Ecuador	b	1	1	-
9	*Sympetrum fonscolombii*	Libellulidae	Azores, Portugal; Sardinia, Italy	b	22	13	9
10	*Trithemis annulata*	Libellulidae	Pontevedra, Spain	b	1	-	1
	Suborder Zygoptera (Damselflies)						
11	*Calopteryx haemorrhoidalis*	Calopterygidae	Italy, Portugal, Spain	b	8	8	-
12	*Ca. splendens*	Calopterygidae	Frosinone, Italy	b	2	2	-
13	*Haetarina* sp.	Calopterygidae	Peru	b	1	1	-
14	*Acanthagrion quadratum*	Coenagrionidae	Xalapa Mexico	b	3	3	-
15	*Aeolagrion* sp.	Coenagrionidae	Pará, Brazil	b	1	1	-
16	*A. axine*	Coenagrionidae	Napo, Ecuador	b	3	3	-
17	*A. inca*	Coenagrionidae	Pacaya-Samiria, Loreto, Peru	b	1	1	-
18	*Argia joergenseni*	Coenagrionidae	Argentina	b	2	2	-
19	*A. kokama*	Coenagrionidae	Tiputini, Ecuador	b	1	1	-
20	*Bromeliagrion* sp.	Coenagrionidae	Pará, Brazil	b	1	1	-
21	*B. fernandezianum*	Coenagrionidae	Tiputini Ecuador	b	1	1	-

22	*B. rehni*	Coenagrionidae	Tiputini, Ecuador	b	1	1	-
23	***Coenagrion puella***	Coenagrionidae	Ness Gardens, Cheshire and Sefton park Merseyside, UK	a	28	12	16
24	***Enallagma cyathigerum***	Coenagrionidae	Ness Gardens, Cheshire and Sefton park, Merseyside, UK	a	7	7	-
25	***Ischnura elegans***	Coenagrionidae	Ness Gardens, Cheshire, UK	a	10	10	-
26	*I. graellsii*	Coenagrionidae	Galicia	b	18	18	-
27	*I. hastata*	Coenagrionidae	Azores (Portugal), Dominican Republic, Jamaica, Cuba, Mexico, Florida	b	43	43	-
28	*Leptobasis vacillans*	Coenagrionidae	Santiago de Cuba, Cuba	b	2	2	-
29	*Metaleptobasis brysonima*	Coenagrionidae	Pará, Brazil	b	1	1	-
30	*M. mauffrayi*	Coenagrionidae	Tiputini, Ecuador	b	3	3	-
31	*M. quadricornis*	Coenagrionidae	Pará, Brazil	b	1	1	-
32	*Phoenicagrion karaja*	Coenagrionidae	Pará, Brazil	b	3	3	-
33	*Telebasis carmesina*	Coenagrionidae	Minas Gerais, Brazil	b	1	1	-
34	*T. dominicana*	Coenagrionidae	Represa Chalons, Cuba	b	3	3	-
35	*T. salva*	Coenagrionidae	Morelos, México	b	2	2	-
36	*Heteragrion bariai*	Megapodagrionidae	Napo, Ecuador	b	1	1	-
37	*Hypolestes clara*	Megapodagrionidae	Jamaica	b	12	12	-
38	*H. hatuey*	Megapodagrionidae	Arroyo Bermejo, Dominican Republic	b	10	10	-
39	*H. trinitatis*	Megapodagrionidae	Cuba	b	10	10	-
40	*Oxystigma* sp.	Megapodagrionidae	Pará, Brazil	b	1	1	-
41	*Philogenia* sp.	Megapodagrionidae	Napo, Ecuador	b	1	1	-
42	*Chalcopteryx rutilans*	Polythoridae	Trocha Quebrada, Peru	b	1	1	-
43	*Cora* sp.	Polythoridae	Panguana, Peru	b	1	1	-
44	*Polythore aurora*	Polythoridae	Iquitos, Peru	b	1	1	-
45	*P. lamerceda*	Polythoridae	Peru	b	3	3	-
46	*P. ornata*	Polythoridae	Pampa Hermosa, Peru	b	6	6	-
47	*P. picta*	Polythoridae	Pozuzo, Peru	b	7	7	-
48	*P. spaeteri*	Polythoridae	Panguana, Peru	b	4	4	-
49	*P. victoria*	Polythoridae	Pozuzo, Peru	b	9	9	-
50	*Drepanoneura sp.*	Protoneuridae	Napo, Ecuador	b	3	3	-
51	*D. muzoni*	Protoneuridae	Tiputini, Ecuador	b	2	2	-

52	*Epipleoneura metallica*	Protoneuridae	Mato Grosso, Brazil	b	3	3	-
53	*E. fuscaenea*	Protoneuridae	Guyana	b	2	2	-
54	*E. humeralis*	Protoneuridae	Tiputini, Ecuador	b	4	4	-
55	*E. machadoi*	Protoneuridae	Mato Grosso, Brazil	b	2	2	-
56	*E. williamsoni*	Protoneuridae	Minas Gerais, Brazil	b	1	1	-
57	*Neoneura* sp.	Protoneuridae	Pará, Brazil	b	2	2	-
58	*N. amelia*	Protoneuridae	Veracruz Mexico	b	1	1	-
59	*N. bilinearis*	Protoneuridae	Guyana	b	1	1	-
60	*N. confudens*	Protoneuridae	Guyana	b	2	2	-
61	*N. denticulata*	Protoneuridae	Pará, Brazil	b	1	1	-
62	*N. joana*	Protoneuridae	Guyana	b	2	2	-
63	*N. myrthea*	Protoneuridae	Guyana	b	2	2	-
64	*N. maria*	Protoneuridae	Cuba	b	3	3	-
65	*N. sylvatica*	Protoneuridae	Mato Grosso, Brazil	b	1	1	-
66	*Phasmoneura* sp.	Protoneuridae	Mato Grosso, Brazil	b	1	1	-
67	*P. exigua*	Protoneuridae	Mato Grosso, Brazil	b	1	1	-
68	*Protoneura* sp.	Protoneuridae	Pará, Brazil	b	1	1	-
69	*P. caligata*	Protoneuridae	Topes de Collantes, Cuba	b	1	1	-
70	*P. capillaris*	Protoneuridae	Dos Bocas, Cuba	b	1	1	-
71	*P. klugi*	Protoneuridae	Tiputini, Ecuador	b	1	1	-
72	*P. sanguinipes*	Protoneuridae	Dominican Republic	b	3	3	-
73	*P. viridis*	Protoneuridae	Jamaica	b	1	1	-
74	*Psaironeura* sp.	Protoneuridae	Pará, Brazil	b	1	1	-
75	*P. tenuissima*	Protoneuridae	Tiputini, Ecuador	b	4	4	-
Additional UK coenagrionid damselflies for the Focussed Screen							
1	***Coenagrion mercuriale***	Coenagrionidae	New Forest, Hampshire, UK	b	30	30	-
2	***C. pulchellum***	Coenagrionidae	Norfolk, UK	b	20	20	-
3	***Ceriagrion tenellum***	Coenagrionidae	New Forest, Hampshire, UK	a	5	-	5
4	***Erythromma najas***	Coenagrionidae	Cheshire, UK	b	5	5	-
5	***Pyrrhosoma nymphula***	Coenagrionidae	Ness Gardens, Cheshire, UK	a	7	7	-

2.3.2 Broad screen in geographic taxa with PCR assays

All DNA samples was quality checked (QC) to confirm that the DNA samples contained amplifiable DNA template after storage/preparation. DNA QC was performed using the mtDNA barcoding primers LCO_2190 / HCO_2198 [242] and C1J_1718 / C1N_2191 [243] (Table 2.2). These primer pairs amplify approximately 680 and 470-bp product size respectively in the Cytochrome oxidase subunit 1 (*COI*) gene region. The cycling conditions are in the legend of Table 2.2.

For samples passing QC, *Rickettsia* presence was assayed using *Rickettsia*-specific primers amplifying a) a section of the bacterial *16S rRNA* gene: Ri170_F / Ri1500_R designed by Küchler *et al*. [169], b) the citrate synthase gene (*gltA)*; RiGltA405_F / RiGltA1193_R designed by Pilgrim *et al*. [175] (Table 2.2). These primers have been shown to amplify across currently known *Rickettsia* groups but not cross amplify other alphaproteobacteria. Cycling conditions were the same as described on Table 2.2. Nuclease free water was used as a negative control to ensure there were no false positive amplifications, and genomic DNA of *Culicoides newsteadi* obtained from Pilgrim *et al*. [175] as a positive control. For each species where a positive amplicon was obtained, amplicons were cleaned of primers and unincorporated nucleotides with ExoSAP-IT kit (E1050, New England Biolabs, US), and Sanger sequenced from a subset of individuals. The sequence was then used (a) to confirm the amplicon was a *Rickettsia* gene product, and (b) to allow estimation of the relatedness of the strains found. These verified positives samples were also used as positive controls in the later screens. All the DNA sequences were deposited in the European Nucleotide Archive (ENA) at EMBL-EBI database.

Table 2.2 PCR primer and fluorescence probe sequences that were used in this study. The rickettsial fluorescence probe is labelled with a fluorophore, in the square bracket, at 3' end. All the primers were used in the following PCR conditions; initial denaturation at 95 °C for 5 min, followed by 35 cycles of denaturation (94°C for 30s), annealing (Tm°C for 30s), extension (72°C for 120s), and a final extension at 72°C for 7 min. The annealing temperature was varied by the primers.

Target organisms: gene	Primer/ probe name	Sequence (5'-3')	Tm (°C)	Product size (bp)	Ref.
Odonates mitochondrial markers					
Cytochrome c oxidase subunit I, ***COI***	*C1J_1718*	GGA GGA TTT GGA AAT TGA TTA GT	52	470	[244]
	C1N_2191	CAG GTA AAA TTA AAA TAT AAA CTT CTC G			
Cytochrome c oxidase subunit I, ***COI***	*LCO_2190*	GGT CAA CAA ATC ATC AAG ATA TTG G	52	680	[242]
	HCO_2198	TAA ACT TCAG GGT GAC CAA AAA ATC A			
Odonates nuclear markers					
Arginine methyltransferase, ***PRMT***	*ARG_F4*	TCG ACT CGT ATG CGC ATT TCG G	52	760	[241]
	ARG_R3	TGC CAC CTT CCT AAT AGA GCT C			
Phosphoglucose isomerase, ***PGI***	*Cp pgi 2191 F*	CTG CTG ACT TCA TAG CCC CTG TAA	56	745	[241]
	Cp pgi 1455 R	GGC CCC WAG AGT AAA AGG TGT GAC			
Myosin light chain, ***MLC***	*Myo_F1u*	ACT TCA CCC AAC TGC TCAC	47	320	[241]
	Myo_R1cm	CAT CAT CGA ATG ACT TGA			
Rickettsia					
16S ribosomal RNA, ***16S rRNA***	*Ri170_F*	GGG CTT GCT CTA AAT TAG TTA GT	54	1.1k	[169]
	Ri1500_R	ACG TTA GCT CAC CAC CTT CAG G			
Citrate synthase, ***gltA***	*RiGltA405_F*	GAT CAT CCT ATG GCA	54	786	[175]
	RiGltA1193_R	TCT TTC CAT TGC CCC			
ATP synthase subunit alpha, ***atpA***	*RiAtpA327_F*	GTC GGT AAA GCA TTG CTT GGT	54	977	[175]
	RiAtpA1309_R	ATT GAT CCT GCT TCA ATA			
Cytochrome c oxidase subunit I, ***coxA***	*RiCoxA317_F*	ATA GGT GCA CCG GAT ATG GC	54	1021	[175]
	RiCoxA1409_R	CCG ATA GAT GAT ACC ATA TTC CA			
Outer membrane protein, ***ompA***	*Ri17kD_F*	TCT GGC ATG AAT AAA CAA GG	54	319	[175]
	Ri17kD_R	ACT CAC GAC AAT ATT GCC C			
***Rickettsia* probe**					
rRNA probe	*RickB1*	CCA TCA TCC CCT ACT ACA-[ATTO 633]	-	-	[10]

2.3.3 Focussed screen in UK coenagrionids with PCR assays

I focussed on 8 coenagrionid species from the UK. Specimens of the 3 UK species, *Coenagrion pulchellum, C. mercuriale* and *Erythromma najas*, were obtained from the previous studies (see section 2.3.1). Five UK coenagrionid species (i.e., *Coenagrion puella, Ceriagrion tenellum, Enallagma cyathigerum, Ischnura elegans* and *Pyrrhosoma nymphula*) were freshly collected from Hampshire, Cheshire and Merseyside areas (Table 2.1). These samples were prepared and screened as described above to obtain *Rickettsia* marker sequences. Additionally, host mitochondrial barcodes were sequenced to confirm species identity, alongside additional nuclear DNA markers to distinguish between the sister species *Coenagrion puella* and C. *pulchellum.* For distinction between *C. puella/pulchellum,* the Myosin light chain (*MLC*), Arginine methyltransferase (*PRMT*) and Phosphoglucose isomerase (*PGI*) genes were amplified and sequenced (Table 2.2), the PCR protocols were adapted from Ferreira *et al.* [241].

2.3.4 Multi-locus sequence typing (MLST) in UK coenagrionids

To allow a more in-depth study of *Rickettsia* diversity in the UK coenagrionid group, *Rickettsia* infections detected were further characterized by sequencing three additional loci; ATP-synthase (*atpA*), 17kDa antigenic protein (*ompA*) and *COI* loci, to create a five loci allelic profile, allowing multi-locus sequence typing (MLST). The PCR conditions of these genes are in the Table 2.2. The PCR primers were designed by Pilgrim *et al.* [175].

2.3.5 Fluorescence in-situ hybridization (FISH)

Evidence for heritable symbiosis was investigated in *C. puella* by using fluorescence *in-situ* hybridization (FISH) to ascertain the presence/absence of *Rickettsia* in ovarian tissues. Methods were adapted from Sakurai *et al*. [12]. Briefly, internal organs of three female *C. puella* (target species, *Rickettsia* positive) and three female *Ischnura elegans* (non-*Rickettsia* infected species) were dissected and fixed in Carnoy's solution (chloroform: ethanol: acetic acid, 6: 3: 1) overnight. Tissues were then cleared with 6% H_2O_2 in ethanol for 12 hr or until the tissue were translucent (whichever was longer). Ovary material was then selected, and hybridization

conducted through incubating the tissues overnight in a hybridization buffer (20mM Tris-HCl pH 8.0, 0.9M NaCl, 0.01% Sodium dodecyl sulphate and 30% formamide) with 10 pmol/ml of rickettsial rRNA specific probe, *rickB1* [10] (Table 2.2). After incubation, tissues were washed in buffer (0.3M NaCl, 0.03 M sodium citrate and 0.01% sodium dodecyl sulphate), mounted onto a slide using VECTASHIELD® Antifade with DAPI (H-1200-10, Vectorlabs, UK) as a mounting medium, and visualised under a confocal microscope, 880 BioAFM (on 880 LSM platform, ZEISS, Germany).

2.3.6 Diversity and relatedness of odonate *Rickettsia*

The phylogenetic relatedness of *Rickettsia* strains found in odonates based on *16S rRNA* and *gltA* genes were estimated using MEGA X [245, 246]. I selected several published sequences of *Rickettsia* from NCBI GenBank, including representatives varying in range from close to far distance relations to the strains in this study, based on BLAST homology. The distant relative group consisted of several vertebrate pathogenic *Rickettsia* and other insect endosymbionts which are known to belong to other clades. *Occidentia massiliensis* was chosen for the outgroup for this *Rickettsia* topology. Sequences were manually checked and aligned using MUSCLE algorithm with default settings [247]. The relationships between these strains were estimated through the Maximum Likelihood approach using MEGA X under the K2+I and T92+G+I model for *16S rRNA* and *gltA* gene, respectively. Support for individual nodes was tested with 1000 bootstrap replicates.

2.4 RESULTS

2.4.1 Prevalence of *Rickettsia* in broad and focussed screen

The initial broad screen of odonate material detected *Rickettsia* amplicons in 8 of the 75 species screened (Table 2.3), which represented 4 of 8 families included in the screening (50% of the screened families were infected). Positive material was derived from UK, South America, Mainland Europe and the Azores, indicating a broad geographic basis to the symbiosis. When observed in-depth in the focused screen, four further *Rickettsia* symbioses were detected in the five additional UK species of Coenagrionidae tested in the focused screening (Table 2.3), resulting in a total of 6 of 8 UK coenagrionids testing positive.

In cases where infection was detected within a species, the fraction of individuals testing positive for *Rickettsia* varied from 9 to 100% (Table 2.4). In two of the species with more than one sample, *C. puella* and *Enallagma cyathigerum*, 100% of the screened individuals were infected. In cases where the individual sex was known (i.e., template derived from adults), there was no evidence of *Rickettsia* infection being biased to one host sex.

Table 2.3 Screening results of *Rickettsia*-positive species from broad screen and focused UK coenagrionids screen (the species that are highlighted in bold). The focused screen combined all coenagrionid species from the broad and additional screened from Table 2.1. In Collection column, 'a' indicates the specimens that were freshly collected in this study, 'b' indicates the samples that were obtained from previous studies (see section 2.3.1). Inside the brackets is the number of screened individuals, and outside is the number of infected individuals

No.	Species	Family	Location	Collection	*N*	Adult	Nymph
Broad Global Screen							
	Suborder Anisoptera (Dragonflies)						
1	*Libellula depressa*	Libellulidae	Ness Gardens, Cheshire, UK	a	1(1)	0	1(1)
2	*Sympetrum fonscolombii*	Libellulidae	Azores, Portugal; Sardinia, Italy	b	2(22)	1(13)	1(9)
	Suborder Zygoptera (Damselflies)						
3	***Coenagrion puella***	Coenagrionidae	Ness Gardens, Cheshire and Sefton park, Merseyside, UK	a	28(28)	12(12)	16(16)
4	***Enallagma cyathigerum***	Coenagrionidae	Ness Gardens, Cheshire and Sefton park, Merseyside, UK	a	7(7)	7(7)	-
5	*Polythore lamerceda*	Polythoridae	Peru	b	1(3)	1(3)	-
6	*P. picta*	Polythoridae	Pozuzo, Peru	b	1(7)	1(7)	-
7	*Drepanoneura muzoni*	Protoneuridae	Tiputini, Ecuador	b	1(2)	1(2)	-
8	*Neoneura sylvatica*	Protoneuridae	Mato Grosso, Brazil	b	1(1)	1(1)	-
Additional UK coenagrionid damselflies for the Focussed Screen							
1	***Coenagrion mercuriale***	Coenagrionidae	New Forest, Hampshire, UK	b	19(30)	19(30)	-
2	***C. pulchellum***	Coenagrionidae	Norfolk, UK	b	15(20)	15(20)	-
3	***Erythromma najas***	Coenagrionidae	Cheshire, UK	b	1(5)	1(5)	-
4	***Pyrrhosoma nymphula***	Coenagrionidae	Ness Gardens, Cheshire, UK	a	4(7)	4(7)	-

Table 2.4 Summary of *Rickettsia* positive species by sex identified across the broad screen and focused screen (the species are highlighted in bold). Where multiple locations specified, the origin of the positive sample is marked with a superscript number indication the number of infections found there. Asterisks indicate those UK coenagrionid species where the *Rickettsia* strains were successfully sequenced for all five MLST loci.

No.	Species	Family	Location	*N*	Adult			Nymph	% infected
					Male	Female	Unknown		
United Kingdom									
1	*Libellula depressa*	Libellulidae	Ness Gardens, Cheshire, UK	1(1)	-	-	-	1(1)	100
2	***Coenagrion puella****	Coenagrionidae	Ness Gardens, Cheshire[18] and Sefton park, Merseyside[10], UK	28(28)	8 (8)	4 (4)	-	16(16)	100
3	***C. pulchellum****	Coenagrionidae	Norfolk, UK	15(20)	-	-	15(20)	-	75
4	***C. mercuriale****	Coenagrionidae	New Forest, Hampshire, UK	19(30)	12 (20)	7 (10)	-	-	63
5	***Enallagma cyathigerum****	Coenagrionidae	Ness Gardens, Cheshire[4] and Sefton park Merseyside[3], UK	7(7)	6(6)	1 (1)	-	-	100
6	***Erythromma najas***	Coenagrionidae	Cheshire, UK	1(5)	1 (5)	-	-	-	20
7	***Pyrrhosoma nymphula****	Coenagrionidae	Ness Gardens, Cheshire, UK	4(7)	4 (7)	-	-	-	57
South America									
8	*Drepanoneura muzoni*	Protoneuridae	Tiputini, Ecuador	1(2)	1 (1)	0 (1)	-	-	50
9	*Neoneura sylvatica*	Protoneuridae	Minas Gerais, Brazil	1(1)	1(1)	-	-	-	100
10	*Polythore lamerceda*	Polythoridae	Peru	1(3)	0 (1)	1 (2)	-	-	33
11	*P. picta*	Polythoridae	Pozuzo, Peru	1(7)	1 (6)	0 (1)	-	-	14
Mainland Europe and the Azores									
12	*Sympetrum fonscolombii*	Libellulidae	Azores, Portugal[2]; Villasimius, Sardegna, Italy	2(22)	0 (6)	1 (4)	0(3)	1(9)	9

2.4.2 Diversity and relatedness of odonate *Rickettsia*

Eleven *Rickettsia* strains from 12 infected odonate species successfully produced *gltA* amplicons, while *16S* amplicons were observed from 9 of 12 infected species. All the sequenced amplicons were used in phylogenetic analysis by reconstructing *16S rRNA* (Figure 2.2) and *gltA* topology (Figure 2.3), except the *Rickettsia* strain from a damselfly *Drepanoneura muzoni* that produced a low quality of DNA sequence for the both genes. The *Rickettsia* infections detected all belong to the torix clade of *Rickettsia*. The infections were diverse, with multiple strains found in odonates, all of them closely allied to *Rickettsia* strains found in other invertebrate taxa.

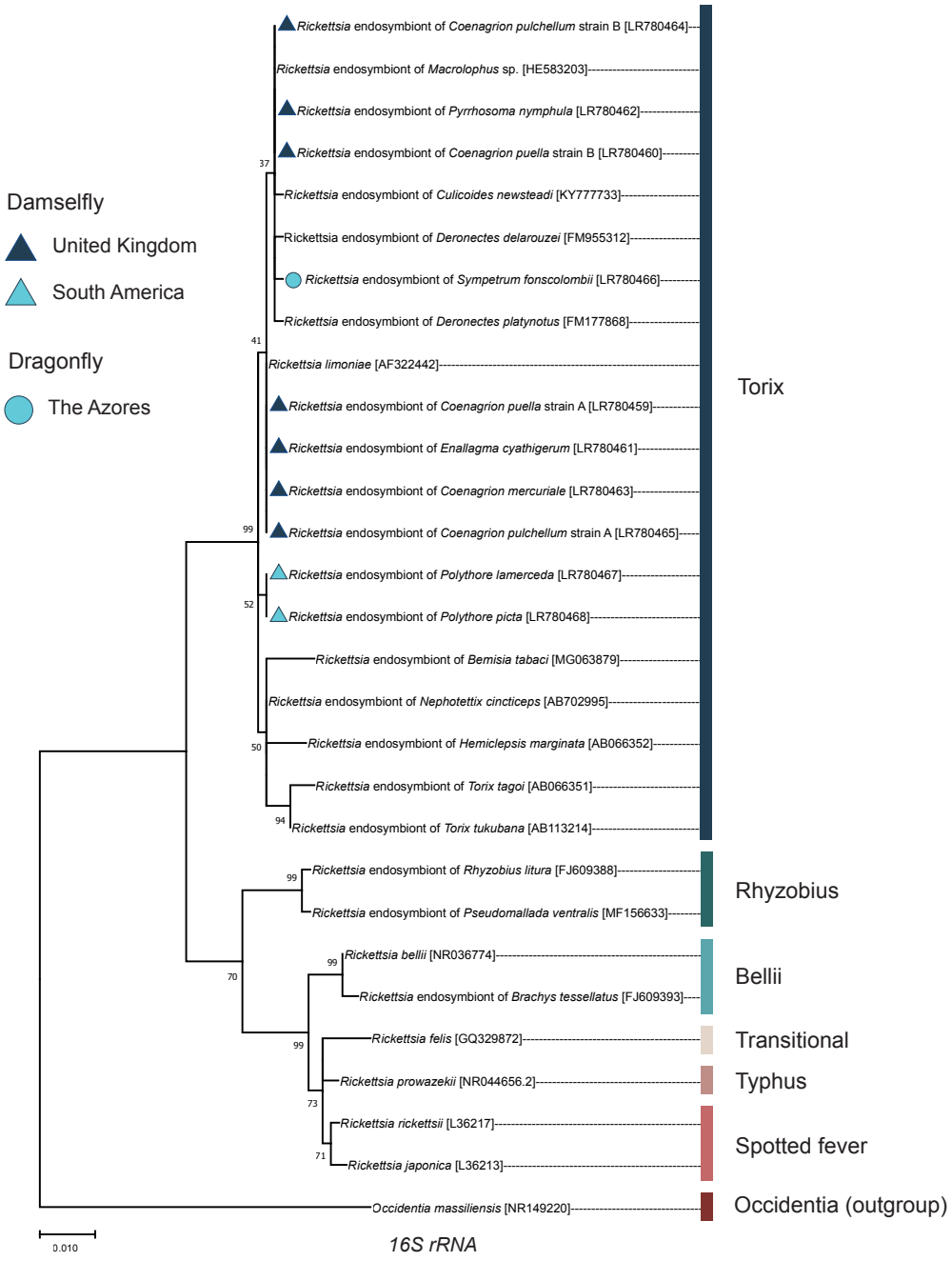

Figure 2.2 Phylogenetic tree of *Rickettsia* based on *16S rRNA* gene. Sequences from screened odonate species, marked with coloured shapes, alongside reference DNA sequences of other *Rickettsia* groups obtained from GenBank (accession numbers in brackets). The tree was constructed in MEGA X by maximum likelihood, with K2+I model. Numbers above branches indicate bootstrap values from 1000 resampling events. Labels indicate the host species from which the symbiont amplicon was obtained.

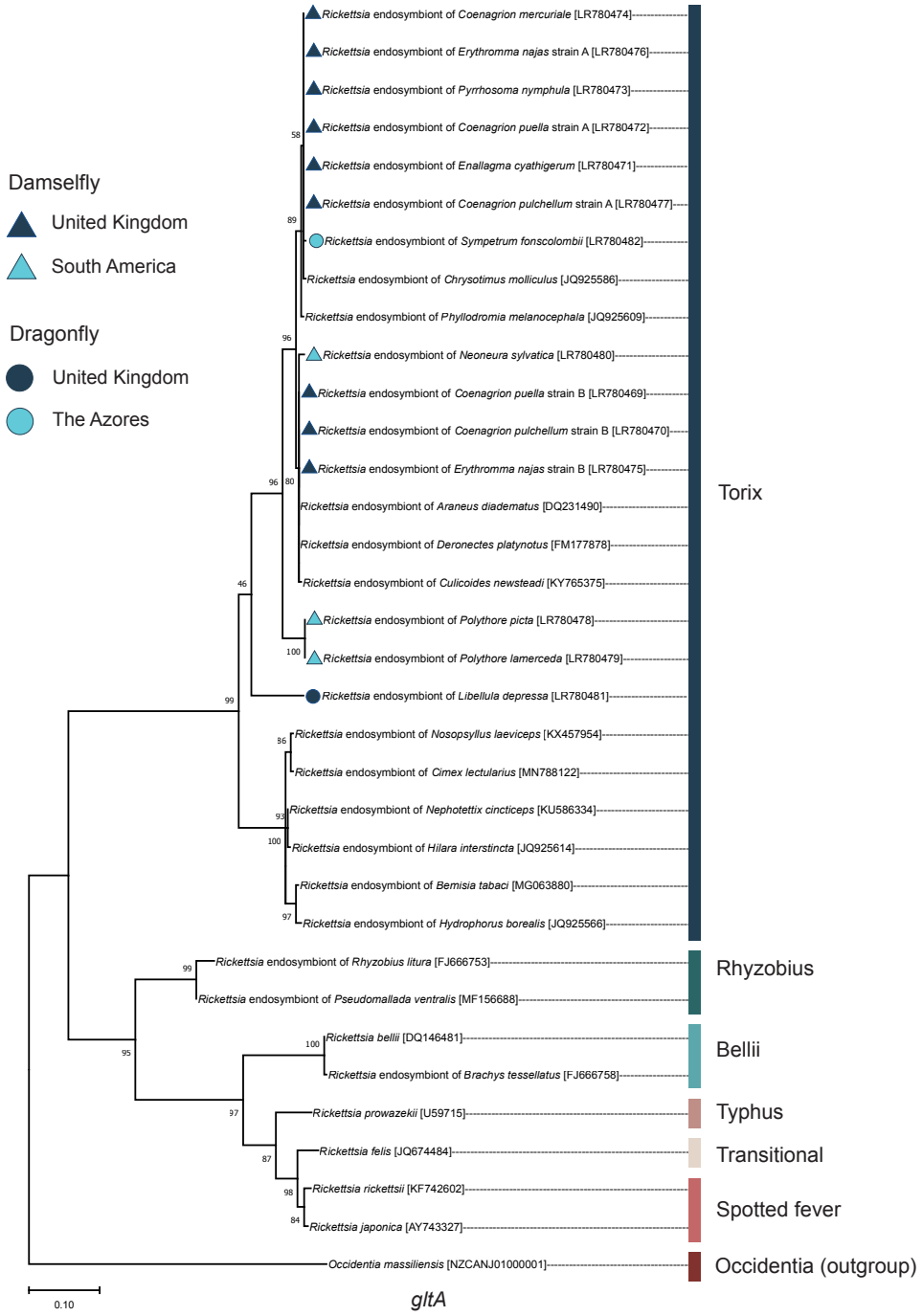

Figure 2.3 Phylogenetic tree of *Rickettsia* based on *gltA* gene. Sequences from screened odonate species, marked with coloured shapes, alongside reference DNA sequences of other *Rickettsia* groups obtained from GenBank (accession numbers in brackets). The tree was constructed in MEGA X by maximum likelihood, with T92+G+I model. Numbers above branches indicate bootstrap values from 1000 resampling events. Labels indicate the host species from which the symbiont amplicon was obtained.

2.4.3 MLST

The MLST study of the UK coenagrionid species infected with *Rickettsia* revealed the presence of four closely related *Rickettsia* strains falling into two clusters, as established in the MLST profiles (Table 2.5). The data also revealed that the sister species *C. puella* and *C. pulchellum*, which share a mtDNA *COI* haplotype (but are distinct at nuclear loci, data not shown), share two *Rickettsia* strains, strain A and B, (Table 2.5). In these two species there was a mix of dual (strain A and B) and single (only strain A) *Rickettsia* infected individual damselflies (Coinfection was observed in five of 10 *C. puella*, and two of three *C. pulchellum*). There were no individuals of either species infected with single *Rickettsia* strain B. Focussed analysis of 10 *C. puella* and 3 *C. pulchellum* individuals revealed an individual was either repeatedly monomorphic, or repeatedly polymorphic, across five loci (five individuals of each type, see Appendix Table S1). The polymorphisms observed were largely at synonymous sites, indicating retained functionality of the gene product.

Table 2.5 MLST allelic profiles of *Rickettsia* from five coenagrionid species in UK. For any gene locus, sequences with the same number are identical. Strain is defined as identity across all loci.

Species	MLST allelic profiles					
	16S rRNA	*gltA*	*ompA*	*atpA*	*coxA*	Strain
Coenagrion puella strain A	1	1	1	1	1	A
C. puella strain B	2	2	2	2	2	B
C. pulchellum strain A	1	1	1	1	1	A
C. pulchellum strain B	2	2	2	2	2	B
C. mercuriale	1	1	1	1	1	A
Pyrrhosoma nymphula	2	3	2	2	2	C
Enallagma cyathigerum	1	1	1	3	1	D

2.4.4 Tropism of torix *Rickettsia*

The tissue-mounted fluorescence *in situ* hybridization revealed a cellular tropism of torix *Rickettsia* in *C. puella.* The signal of *Rickettsia* (ATTO-633 fluorophore) was detected throughout the ovary tissues of *C. puella*, mostly in the nuclei and cytoplasmic area of both mature and early developing oocytes, while the signal was absent in the non-infected species, *I. elegans* (Figure 2.4).

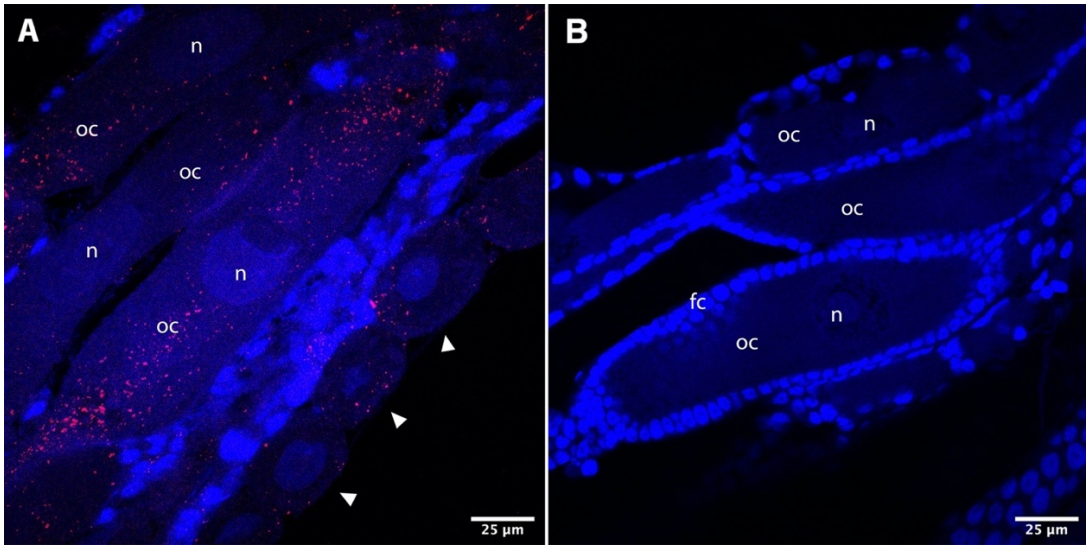

Figure 2.4 Fluorescence in situ hybridization (FISH) images of damselfly ovaries. FISH showing the localisation of torix *Rickettsia* in **A** *Coenagrion puella* (*Rickettsia* positive) and **B** *Ischnura elegans* (*Rickettsia* negative) oocytes. Red colour (ATTO633 label) represents *Rickettsia* signal and blue areas (DAPI) damselfly nuclei. Infection is observed throughout the ovary tissue of *C. puella*, mostly in oocytes (oc) and early differentiated oocytes (white arrowhead), but no signal of the symbiont was observed in the ovary of the *Rickettsia*-negative species, *I. elegans*; fc, follicular epithelial cells; n, nucleus of oocyte.

2.5 DISCUSSION

There are numerous heritable microbe taxa that circulate in insects which play important roles as partners and antagonists. While the majority of studies have focused on the 'global pandemic' of *Wolbachia* and its consequences for host biology, ecology and evolution [248]; other heritable symbionts remain less well studied, particularly in freshwater insects. Here, I examined odonates for just one such symbiont – torix group *Rickettsia*.

2.5.1 The 'big picture' of torix *Rickettsia* in odonates

Within the global screen, *Rickettsia* was found in 8 of 75 odonate species (10.7%) and for the focussed UK screen, and in 6 of 8 (75%) species from the coenagrionid family. The *Rickettsia* infections discovered all fall into the torix group, a basal group of *Rickettsia* with high levels of diversity, previously highlighted as common in other aquatic invertebrates [135, 169, 175]. The fraction of infected species in this screen is likely to be an underestimate as there are two systematic biases likely to produce false negative results. First, symbiont infections vary in prevalence within species, and can infect a minority of individuals. The limited number of individuals tested for some of the species screened will miss some species with low or intermediate levels of infection. Second, the material available for testing was commonly derived from legs. Symbiont infection that is strongly localised within a host individual (and not present in hemocytes) will appear as negative when leg material is screened. Thus, an estimation of the fraction of infected species will be an underestimate. Further, although this data record of more infections in species of UK coenagrionids than elsewhere, could also be a product of a greater sampling intensity. What is clear, however, is that whilst odonates are hosts to *Rickettsia*, and they carry torix group strains like other freshwater invertebrates [175].

2.5.2 The limits of torix *Rickettsia* screening

The study of torix *Rickettsia*/insect symbioses is a relatively young field of research, with this diverse group only first described in 2002 [9]. Thus, despite now being known to be widespread, data on the biology of these symbioses is absent or

extremely limited. For instance, within-host titres are unknown, meaning I do not know how many cells have to be present to be able to detect an infection. However, *Rickettsia* distribution in insect tissues is commonly diffuse, including haemocytes, Malpighian tubules, gut lining, and in oocytes, where they seem to invade through the follicular epithelium and, unusually, they have also been found in sperm [249].

2.5.3 Diversity of *Rickettsia* at spatial scale

The symbioses in this study were found in representative species from the two odonate suborders: Zygoptera (damselfly) and Anisoptera (dragonfly). These species belong to four different families and derive from both Europe and South America (Tables 2.3 and 2.4). Sequence analysis revealed a wide diversity in *Rickettsia* infections and are not monophyletic within Odonata, suggesting the *Rickettsia*-odonate symbiosis has multiple origins. The odonate *Rickettsia* grouped together with strains found in other host species, e.g., *Deronectes* water beetle, *Araneus* orb-weaving spider, *Culicoides* biting midge and *Cimex* common bedbug (Figure 2.2 and 2.3). There also appeared to be a hot-spot in UK coenagrionids, in which four MLST strains from two clusters were observed, with two of these strains present in several species. The MLST study of *Rickettsia* is a recent initiative, introduced by Pilgrim *et al*. in 2017 [175]. Therefore, more fine scale comparisons between the *Rickettsia* strains in this chapter with those found in other insect orders are limited in scope, due to lack of multi locus data from other taxa. However, this geographically confined clade may reflect symbiont movement between co-occurring odonate species or derivation from a common local source [224].

2.5.4 Double infections in the two sister coenagrionids

The presence of double peaks in sequences of *Rickettsia* marker genes in *C. puella* and *C. pulchellum* provide evidence of coinfection, where a single individual carried two strains of *Rickettsia*. Individuals either show one sequence of strain A at all markers, or two sequences mixing of strain A and B at all markers (with two strains identified). Variable loci can either be the product of two infecting symbiont strains, or a single symbiont alongside a symbiont genome insertion into the insect chromosome [250]. That the amplicons represent two symbionts, rather than a

symbiont and a nuclear insertion of symbiont genetic material, is implied by the nature of the variants. The majority of variable sites observed are synonymous differences (e.g., in *GltA* gene has 16 SNP in 715 bps, of which 14 are synonymous and 2 non-synonymous), that indicate retained functionality of the gene (Appendix Table S1). Retained functionality is expected for a symbiont copy (where function is required) rather than a nuclear insert (which is expected to pseudogenize). Coinfections are well known for *Wolbachia* [251] but are less commonly recorded for other symbionts; however, they are clear in this system.

2.5.5 Torix *Rickettsia* as a cause of mtDNA introgression

Within the UK group, I observed a pair of *Rickettsia* strains shared by the sister species pair *C. puella* and *C. pulchellum*. This species pair is robustly supported in analysis of nuclear markers [220, 241] but shares a mtDNA barcode [224]. Shared mtDNA barcodes for otherwise distinct species pairs commonly reflects introgression of the mtDNA across the species boundary [228]. This process is known to be driven by *Wolbachia* in other cases [252, 253]. Whilst hybridization is considered very uncommon between these species [225], mitochondrial introgression requires only a single hybridization event, and it is likely that both the shared mtDNA and symbiont in this case reflect a history of symbiont movement across the species barrier, along with accompanying mtDNA. This process produces distinct species, divergent at nuclear markers, that then have no mtDNA 'barcoding gap', as observed in the case of *C. puella* and *C. pulchellum*. An immediate implication of these results is that screening for *Wolbachia* alone is not sufficient to rule out symbiont-mediated introgression of mtDNA.

2.5.6 The evidence of maternal inheritance

Torix *Rickettsia* are considered likely to show maternal inheritance, and in some cases also show paternal transmission [249]. In this system, *Rickettsia* were visible in *C. puella* ovarioles under FISH microscopy, making maternal inheritance very likely. Additionally, infection was detected in both larvae and adults, which implies vertical transmission (Table 2.4). Thus, these data supports the idea that *Rickettsia* is a heritable symbiont in odonates, as inferred for other taxa [144, 150, 175, 249].

The significance of the symbiosis is uncertain. Vertical transmission through eggs ties *Rickettsia* transmission to odonate survival and reproduction, and thus selects for symbiont contribution to host function [117]. Heritable symbiont are commonly important contributors to organismal function but the impact of torix *Rickettsia* on their host is poorly understood. However, sex-ratio distortion mediated by *Rickettsia* is unlikely in odonates, as there were no obvious male/female host biases in *Rickettsia* presence in species where large numbers of individuals were collected. Indeed, the symbionts were absent in the only odonate species known to have thelytokous parthenogenesis (*Ischnura hastata* from the Azores islands) [216]. These data, by exclusion, indicate that symbionts are likely retained in odonate hosts by some other means, which should be explored further.

2.6 CONCLUSION

This chapter revealed the first incidence of *Rickettsia* infection in insects of Odonata. All the odonate-associated *Rickettsia* were affiliated to the torix clade with diverse strains, found across dragonflies and damselflies over three geographic regions. Damselflies of the family Coenagrionidae from Great Britain were observed as a hot-spot for the endosymbiont. FISH imaging revealed torix *Rickettsia* were present in oocytes of the azure damselfly (*Coenagrion puella*). It can be assumed that the symbionts are inherited via a vertically transmitted route. The evidence of double *Rickettsia* strain infections with the same pattern in the sister species *C. puella* and *C. pulchellum* indicated that torix *Rickettsia* could have driven mtDNA introgression between the two species. This could represent a further symbiont that can disrupt mtDNA barcoding studies. Finally, not the majority of odonate species are infected with torix *Rickettsia*, but this study demonstrated that torix *Rickettsia* have already established with this freshwater associated insect group and this may support the hypothesis that freshwater invertebrates are a hot-spot for torix *Rickettsia*. Observations for torix *Rickettsia* in a broad spectrum of invertebrate host species from freshwater and terrestrial communities may help to test this hypothesis.

CHAPTER 3

Are freshwater invertebrates a hot-spot for torix *Rickettsia* symbionts?

Publication and author contributions

All data in this chapter has contributed to a publication with Dr Jack Pilgrim as first author, University of Liverpool, in *GigaScience* with a pre-print on Authorea under Pligrim *et al*. [254] Incidence of torix *Rickettsia* in *Anopheles plumbeus* was discovered in PCR assays by Dr Jack Pilgrim from his collections. All DNA extraction, PCR screen and data analysis within this chapter were performed by me.

3.1 ABSTRACT

Torix *Rickettsia* were first described from *Torix targoi* leeches. The records of torix *Rickettsia* since this time led to the generation of the hypothesis that torix *Rickettsia* are more common and widespread in freshwater than terrestrial invertebrates. In this chapter, I present a test of this hypothesis, screening a range of freshwater and terrestrial species for torix *Rickettsia* using PCR assays, with confirmation of putative positive samples through amplicon sequence. Nine strains of torix *Rickettsia* were detected in 57 species of freshwater species, compared to 8 strains found in 112 terrestrial species of invertebrates. Statistical analysis supported the freshwater hot-spot hypothesis. However, some terrestrial taxa – like spiders – also harbour torix *Rickettsia* commonly. The potential drivers of this pattern are discussed.

3.2 INTRODUCTION

In the previous chapter, I observed that around 10% of odonate species carried torix *Rickettsia*. This group of insects is abundant in almost all freshwater habitats. Some strains of the symbionts that were closely related were from different geographical origins, or even from different insect species. This indicated that *Rickettsia* is distributed among and moves between odonate species, and probably between odonates and other taxa. This scenario indicates the possibility of spread more widely amongst members of the aquatic invertebrate community.

3.2.1 Non-pathogenic *Rickettsia* in insects

Whilst *Wolbachia* has represented the focus of insect-endosymbiont research, *Rickettsia* are also found frequently in many insects and other invertebrate species. Originally considered as infectious agents vectored by arthropods [255], *Rickettsia* are generally now recognised as endosymbionts of insects that influence hosts reproduction and biological fitness [57, 89, 110, 135]. *Rickettsia* heritable symbiosis of with insects was first documented in a study in the late of 20th century [57], as the causal agent of the male-killing phenotype in the two-spotted ladybird (*Adalia bipunctata*). The strain of this *Rickettsia* was later named Adalia and established as a group that covered a few related strains thought to exhibit the male killing phenotypes in other beetle species [57]. The current view of *Rickettsia* diversity includes multiple groups, e.g., bellii, meloidae, rhyzobius, transitional and torix [144, 150, 182]. For some of these, there is a good account of the *Rickettsia*-host interaction, but in many cases, our knowledge remains sparse [144].

3.2.2 Torix group *Rickettsia*

The increased number of *Rickettsia* studies reported in recent decades has produced a wider appreciation of this group of bacteria and the invertebrates that harbour them [144]. Amongst these publications, many groups of *Rickettsia* have been observed, but there has been a recent appreciation that many of the symbioses lie in the 'torix group', a group originally found in leech and a few other freshwater organisms [9, 150]. Neither their host preferences nor biological impacts of the

symbionts themselves have been comprehensively studied whereas the number of recorded symbioses involving torix group strains are growing [135, 144, 175, 197].

Currently, the hosts of torix *Rickettsia* are mainly organisms from freshwater ecosystems, e.g., protists [164], hirudineans [9, 135], amphipods [256] and other aquatic insects [175, 197]. On the other hand, some terrestrial hosts are also known, e.g., diplopods [194], arachnids [143, 186] and terrestrial insects [11, 12, 257], though these are considered to be in a minority. This has led to the 'aquatic hot-spot' hypothesis [182], that torix *Rickettsia* might be more abundant in freshwater than the terrestrial communities. However, this hypothesis has not yet been clearly investigated through an unbiased screen comparing between the two environments.

3.2.3 Aims

In this study I completed a survey of the prevalence of torix *Rickettsia* in various invertebrates from the freshwater and the terrestrial community. Infection with symbionts was determined through PCR assays and the strain of infection identified by Sanger sequencing of marker genes. The results of this screen were used to test the freshwater hot-spot hypothesis. Further to this, the phylogenetic relatedness of the *Rickettsia* strains was estimated, to investigate whether there is greater sharing of symbiont strains within communities (freshwater or terrestrial) than between.

3.3 METHODS

3.3.1 Invertebrates collections

Whole specimens of freshwater and terrestrial invertebrates were obtained from direct sampling and contributor collections. For direct sampling, invertebrates were collected from the Kielder Forest and Ness Botanical Garden in late 2016 – 2017 and from the New forest in 2017 (Table 3.1 and 3.2). Terrestrial invertebrates were collected by random sweep from grass and bushes using a sweep net (12" diameter). Freshwater invertebrates were sampled from ponds and small steams, using a pond dipping net (25x25 cm with 1 mm mesh size). All specimens were preserved in 100% EtOH and brought back to the laboratory for identification.

Morphological identification was used to initially classify these animals to species level; if not, at least a family group or a genus group would be noted. However, some species were noted as 'unknown sp.' if they could not be identified from morphological and molecular identification (see section 3.3.2). Several identified freshwater specimens were additionally contributed by Craig Macadam, Bug life Conservation Director. These specimens were preserved and sent in 15 ml tubes filled with 100% EtOH, separated by each species/ tube. Several non-biting midges and a mosquito (*Anopheles plumbeus*) were contributed by Dr Jack Pilgrim, University of Liverpool (Table 3.1). All the specimens were preserved in 100% EtOH and kept at 4^{o}C in a dark cold room until DNA extraction was processed.

Specimens were rinsed with 100% EtOH and then air dried before the DNA extraction. Some specimens were initially dissected for abdomen when the body was thick, or the body length was longer than 0.5 mm (Table 3.1 and 3.2). For invertebrate specimens where the body size was smaller than 0.5 mm, the whole body was used for DNA extraction. Genomic DNA was extracted using Promega Wizard DNA purification kit (A1120, Promega, UK), adapted from the manufacturer protocol (see section 2.3.1 in Chapter 2). DNA pellets were resuspended in molecular graded water and kept at -20^{o}C until use.

Additionally, invertebrate samples in DNA extracts from the study of Duron *et al*. in 2008 [188], which have been held in University of Liverpool laboratory at -80 °C, were used in this study. His collections were from the UK and Europe and were strongly biased to terrestrial invertebrates (101 species were terrestrial, Table 3.2, and only 14 species were freshwater invertebrates, Table 3.1).

Species were defined as 'freshwater' either if the species was retrieved from within the freshwater biome, or if the species is known to have an aquatic phase within the life cycle or live in semi aquatic ecosystems (e.g., wet soil and mosses). Other species were defined as 'terrestrial'.

Table 3.1 Freshwater invertebrate species that were screened in this study. The species are separated in groups based on their classification nomenclature. They were collected from different times and localities. DNA were either extracted from the whole body (w) or dissected abdomen (d). In collection, 'a' indicates the species were collected in fresh specimens in this study, 'b' indicates the species in DNA extracts that were obtained from Duron *et al*. [188].

	Invertebrate groups and Species	Location	Year	*N*	Source of DNA	Collection
Ephemeroptera						
1	*Baetis muticus*	Stirling, Scotland, UK	2017	3	w	a
2	*B. rhodani*	Stirling, Scotland, UK	2017	3	w	a
3	*Cloeon dipterum*	Ness Gardens, Cheshire, UK	2016	3	w	a
4	*Ecdyonurus* sp.1	Stirling, Scotland, UK	2017	5	d	a
5	*Ecdyonurus* sp.2	Ness Gardens, Cheshire, UK	2016	3	d	a
6	*E. venosus*	Ness Gardens, Cheshire, UK	2016	6	d	a
7	*Leptophlebia vespertina*	New Forest, Hampshire, UK	2016	1	w	a
8	*Paraleptophlebia submaginata*	Stirling, Scotland, UK	2017	3	w	a
9	*Rhithrogena semicolorata*	Stirling, Scotland, UK	2017	3	w	a
Trichoptera						
10	*Hydropsyche* sp.	Stirling, Scotland, UK	2017	3	d	a
11	*Polycentropus flavomaculatus*	Ness Gardens, Cheshire, UK	2017	3	d	a
12	*Rhyacophila dorsalis*	Stirling, Scotland, UK	2017	3	d	a
Plecoptera						
13	*Amphinemura sulcicollis*	Stirling, Scotland, UK	2017	3	d	a
14	*Dinocras cephalotes*	Stirling, Scotland, UK	2017	3	d	a
15	*Isoperla grammatica*	Stirling, Scotland, UK	2017	3	d	a
16	*Perla bipunctata*	Stirling, Scotland, UK	2017	3	d	a
Hemiptera						
17	*Corixa punctata*	Ness Gardens, Cheshire, UK	2016	1	d	a
18	*Gerris sp.*	Montferrier sur Lez, France	2006	12	w	b
19	*G. thoracicus*	Ness Gardens, Cheshire, UK	2016	1	d	a
20	*Hydrometra stagnorum*	Montferrier sur Lez, France	2006	20	w	b
21	*Nepa cinerea*	Montferrier sur Lez, France	2006	3	w	b
22	*Notonecta glauca*	Ness Gardens, Cheshire, UK	2016	2	d	a
23	*Plea minutissima*	Notre Dame de Londres, France	2006	8	w	b
24	*Sigara lateralis*	Notre Dame de Londres, France	2006	6	w	b
25	*S. striata*	Ness Gardens, Cheshire, UK	2006	2	d	a
Diptera						
26	*Aedes* sp.	Ness Gardens, Cheshire, UK	2017	8	w	a
27	*A. albopictus*	Roma, Italy	2005	20	w	b

28	*Anopheles plumbeus*	Chester Zoo, Cheshire, UK	2017	2	w	a
29	Chironomidae sp.	Ness Gardens, Cheshire, UK	2016	4	w	a
30	*Chironomus* sp.	Ness Gardens, Cheshire, UK	2016	4	w	a
31	*C. acidophilus*	Ness Gardens, Cheshire, UK	2017	1	w	a
32	*C. plumosus*	Notre Dame de Londres, Franc	2006	20	w	b
33	*Culex pipiens (ssp. quinquefasciatus)*	Puerto Viejo de Talamanca, Costa Rica	2006	20	w	b
34	*C. pipiens pipiens*	St Nazaire de Pézan, France	2006	20	w	b
35	*Eristalinus* sp.	Cheshire, UK	2016	3	d	a
36	*Eristalis tenax*	Montpellier (grotte du zoo), France	2002	7	w	b
37	*Glyptotendipes* sp.	Ness Gardens, Cheshire, UK	2016	1	w	a
38	*Hilara sp.*	Sefton park, Merseyside, UK	2017	3	w	a
39	*Simulium aureum*	New Forest, NewHampshire, UK	2017	1	w	a
40	*S. ornatum*	N/A	2003	12	w	b
41	*Tipula* sp.	UK	2006	10	w	b
42	*T. oleracea*	UK	2006	13	w	b
43	*Zavrelimyia* sp.	Kielder Forest, Northumberland, UK	2017	1	w	a
Coleoptera						
44	*Agabus bipustulatus*	Ness Gardens, Cheshire, UK	2017	3	d	a
45	*Guignotus pusillus*	Notre Dame de Londres, France	2006	12	w	b
46	Unknown sp.1	Ness Gardens, Cheshire, UK	2017	2	w	a
47	Unknown sp.2	Ness Gardens, Cheshire, UK	2017	3	w	a
Acarina						
48	Unknown sp.	Ness Gardens, Cheshire, UK	2017	3	w	a
Isopoda						
49	*Asellus aquaticus*	Ness Gardens, Cheshire, UK	2016	3	d	a
Amphipoda						
50	*Gammarus pulex*	Stirling, Scotland, UK	2017	3	d	a
51	*Crangonyx pseudogracilis*	Ness Gardens, Cheshire, UK	2016	6	d	a
Gastropoda						
52	*Radix balthica*	Ness Gardens, Cheshire, UK	2016	3	w	a
53	*Planorbis* sp.	Ness Gardens, Cheshire, UK	2016	3	w	a
55	*Galba truncatula*	Laboratory in University of Liverpool, Merseyside, UK	2017	20	w	a
Hirudinea						
55	*Erpobdella octaculata*	Ness Gardens, Cheshire, UK	2016	2	d	a
56	*Hemiclepsis marginata*	Ness Gardens, Cheshire, UK	2017	1	d	a
Tricladida						
57	Unknown sp.	Ness Gardens, Cheshire, UK	2016	1	w	a

Table 3.2. Terrestrial invertebrate species that were screened in this study. The species are separated in groups based on their classification nomenclature. They were collected from different times and localities. DNA were either extracted from the whole body (w) or dissected abdomen (d). In collection, 'a' indicates the species were collected directly for this study, 'b' indicates the species in DNA extracts that were obtained from Duron *et al*. [188].

	Invertebrate groups and Species	Location	Year	*N*	Source of DNA	Collection
Araneae						
1	*Agelenopsis aperta*	Tennessee	N/A	12	w	b
2	*Alopecosa pulverulenta*	Berne, Germany	N/A	16	w	b
3	*Amaurobius fenestralis*	Montpellier, France	2006	16	w	b
4	*Araneus diadematus*	Beerse, Belgium	N/A	19	w	b
		Greater London, UK	N/A	8	w	b
5	*Argiope bruennichi*	Hamburg, Germany	N/A	7	w	b
6	*A. lobata*	Spain	N/A	7	w	b
		Israel	N/A	4	w	b
7	*Cyclosa conica*	Brandenburg, Germany	N/A	11	w	b
8	*Dysdera crocata*	Montpellier, France	2006	2	w	b
9	*Enoplognatha ovata*	Greater London, UK	N/A	20	w	b
10	*Erigone arta*	Ness Gardens, Cheshire, UK	2017	1	w	a
11	*Evarcha falcata*	Beerse, Belgium	N/A	5	w	b
12	*Holochnemus pluchei*	Montpellier, France	2006	7	w	b
13	*Hylyphantes graminicola*	Ness Gardens, Cheshire, UK	2017	1	w	a
14	*Larinioides cornutus*	Greater London, UK	N/A	6	w	b
15	*L. sclopetarius*	Hamburg, Germany	N/A	17	w	b
16	*Linyphia triangularis*	Berlin, Germany	N/A	9	w	b
		Greater London, UK	N/A	6	w	b
17	*Lycosa* sp.	Ness Gardens, Cheshire, UK	2017	2	d	a
18	*Metellina mengei*	Greater London, UK	N/A	13	w	b
19	*M. segmentata*	Brandenburg, Germany	N/A	9	w	b
20	*Neriene clathrata*	Beerse, Belgium	N/A	13	w	b
21	*N. peltata*	Ness Gardens, Cheshire, UK	2017	1	w	a
22	*Pachygnatha degeeri*	Berne, Germany	N/A	11	w	b
23	*P. listeri*	Beerse, Belgium	N/A	17	w	b
24	*Pardosa lugubris*	Darmstadt, Germany	N/A	20	w	b
25	*P. pullata*	Brandenburg, Germany	N/A	20	w	b
26	*P. purbeckensis*	Belgium	N/A	19	w	b
27	*Pholcus phalangoides*	Berlin, Germany	N/A	20	w	b
28	*Pisaura mirabilis*	Greater London, UK	N/A	12	w	b

29	*Tetragnatha sp.*	New Forest, Hampshire, UK	2017	3	d	a
30	*T. montana*	Greater London, UK	N/A	20	w	b
31	*Xysticus cristatus*	Cambridgeshire, UK	N/A	16	w	b
32	Unknown sp.	Ness Gardens, Cheshire, UK	2017	2	w	a
Opiliones						
33	*Leiobunum rotundum*	Feurs, France	2006	6	w	b
Ixodida						
34	*Ixodes uriae*	Hornøya, Norway	2005	19	w	b
35	*Rhipicephalus microplus*	New Caledonia, France	2003	1	w	b
Scorpiones						
36	*Euscorpius flavicaudis*	St Nazaire de Pézan, France	2006	1	NA	b
Diplopoda						
37	*Ommatoiulus* sp.	Ness Gardens, Cheshire, UK	2016	1	d	a
Neuroptera						
38	Unknown sp.	Ness Gardens, Cheshire, UK	2017	1	d	a
Mecoptera						
39	*Panorpa* sp.	Ness Gardens, Cheshire, UK	2017	2	d	a
Orthoptera						
40	*Calliptamus italicus*	Notre Dame de Londres, France	2016	18	NA	b
41	*Chorthippus brunneus*	UK	2006	20	NA	b
42	*Gryllomorpha dalmatina*	Montpellier, France	2006	2	NA	b
Blattaria						
43	*Loboptera decipiens*	Montpellier, France	2006	17	NA	b
Mantodea						
44	*Iris oratoria*	St Nazaire de Pézan, France	2006	6	NA	b
45	*Mantis religiosa*	Feurs, France	2006	3	NA	b
Dermaptera						
46	*Forficula auricularia*	Feurs, France	2006	9	w	b
Hemiptera						
47	*Aphis fabae*	Montpellier, France	2006	12	w	b
48	*A. nerii*	Montpellier, France	2006	8	w	b
49	*Baizongia pistaciae*	Viols le Fort, France	2006	12	w	b
50	*Cicadella viridis*	L'Olme, France	2006	16	w	b
51	*Cimex lectularius*	Yorkshire, UK	2008	12	w	b
52	*Elasmucha grisea*	Greater London, UK	2006	16	w	b
53	*Graphosoma italicum*	Montpellier, France	2006	12	w	b
54	*Lygaeus equestris*	Montpellier, France	2006	12	w	b
55	*Notostira elongata*	L'Olme, France	2006	11	w	b
56	*Pyrrhocoris apterus*	Montpellier, France	2006	11	w	b
57	*Rhyparochromus vulgaris*	Castelnaudary, France	2006	20	w	b

Coleoptera						
58	*Anaspis frontalis*	Hérault, France	2004	12	w	b
59	*Anthaxia* sp.	Mont Barri, France	2004	16	w	b
60	*A. nitidula*	Mont Barri, France	2004	20	w	b
61	*Calvia quattuordecemguttata*	Greater London, UK	2006	6	w	b
62	*Capnodis tenebrionis*	Montpellier, France	2006	1	w	b
63	*Cetonia aurata*	Feurs, France	2006	3	w	b
		Mont Barri, France	2004	12	w	b
64	*Chrysolina varians*	Mont Barri, France	2004	18	w	b
65	*Clytus arietis*	Mont Barri, France	2004	20	w	b
66	*Dermestes* sp.	Mont Barri, France	2004	20	w	b
67	*D. tessellatocollis*	Liverpool City Centre, Merseyside, UK	2016	2	w	a
68	*Gastrophysa* sp.	Greater London, UK	2006	20	w	b
69	*Geotrupes stercorarius*	Mont Barri, France	2004	3	w	b
70	*Larinus scolymi*	Aldira de Irmeros, Spain	2005	12	w	b
71	*Leptinotarsa decemlineata*	Feurs, France	2006	10	w	b
72	*Mordellistena* sp.	Mont Barri, France	2004	10	w	b
73	*Oedemera* sp.	Mont Barri, France	2004	20	w	b
74	*Oncocerna* sp.	Mont Barri, France	2004	20	w	b
75	*Pseudovadonia livida*	Mont Barri, France	2004	19	w	b
76	*Phyllobius argentatus*	Mont Barri, France	2004	15	w	b
77	*Stenopterus* sp.	Mont Barri, France	2004	20	w	b
Diptera						
78	*Braula coeca*	Ouessant, France	2002	4	w	b
79	*Chorisops tunisiae*	Montpellier, France	2003	8	w	b
80	*Delia antiqua*	N/A	N/A	11	w	b
81	*D. platura*	N/A	N/A	11	w	b
82	*D. radiacum*	N/A	N/A	10	w	b
83	*Gasterophilus intestinalis*	France	N/A	10	w	b
84	*Hippobosca equina*	Restinclières, France	2006	15	w	b
85	*Lonchoptera lutea*	Ness Gardens, Cheshire, UK	2017	3	w	a
86	*Medetera petrophila*	St Bauzille de Putois, France	2003	12	w	b
87	*Musca domestica*	L'Olme, France	2006	20	w	b
88	*M. vitripennis*	Notre Dame de Londres, France	2003	8	w	b
89	*Neomyia cornicina*	Notre Dame de Londres, France	2003	8	w	b
90	*Protocalliphora* sp.	Corse, France	2003	2	w	b
91	*P. azurea*	Montpellier, France	2005	12	w	b
92	*Psila rosae*	N/A	N/A	11	w	b
93	*Stomoxys calcitrans*	Le Malzieu, France	2001	11	w	b

Lepidoptera						
94	*Chilo phragmitella*	Feurs, France	2006	10	w	b
95	*Euplagia quadripunctaria*	Feurs, France	2006	2	w	b
96	*Pieris brassicae*	Feurs, France	2006	7	w	b
97	*Plodia interpunctella*	Montpellier, France	2006	12	w	b
98	*Thymelicus lineola*	Greater London, UK	2006	15	w	b
99	*T. sylvestris*	Greater London, UK	2006	2	w	b
100	*Triodia sylvina*	Montpellier, France	2006	4	w	b
Hymenoptera						
101	*Amblyteles armatorius*	St Nazaire de Pézan, France	2006	1	w	b
102	*Amegilla albigena*	St Nazaire de Pézan, France	2006	13	w	b
103	*A. ochroleuca*	St Nazaire de Pézan, France	2006	3	w	b
104	*Anthidium florentinum*	St Nazaire de Pézan, France	2006	6	w	b
105	*Apis mellifera*	UK	2006	9	w	b
106	*Bombus terrestris*	NW, Switzerland	2006	20	w	b
107	*Diplolepis rosae*	L'Olme, France	2006	2	w	b
108	*Formica lugubris*	UK	2006	10	w	b
109	*Pachycrepoideus* sp.	UK	N/A	94	w	b
110	*Polistes dominula*	St Nazaire de Pézan, France	2006	4	w	b
111	*P. nimpha*	St Nazaire de Pézan, France	2006	19	w	b
112	*Sceliphron caementarium*	St Nazaire de Pézan, France	2006	3	w	b

3.3.2 PCR assays for torix *Rickettsia*

To investigate the infection of torix *Rickettsia* in invertebrates, DNA templates were first tested for amplifiable DNA quality using the invertebrate mtDNA markers (LCO_1490/HCO_2198 [242] and C1J_1718/C1N_2191 [243] primers) in a conventional PCR assays as described in Chapter 2 (Table 2.2). The amplicons from COI regions were cleaned with ExoSAP-IT kit (E1050, New England Biolabs, US) and Sanger sequenced. These mitochondrial haplotypes were used to identify and confirm the invertebrate species by searching the sequences against the DNA barcodes database from GenBank, NCBI, under BLAST algorithm.

Samples passing this QC were then tested for *Rickettsia* in conventional PCR assays using rickettsial-specific primers base on 16S ribosomal RNA (*16S rRNA*) and citrate synthase subunit A (*gltA)* gene as described in Chapter 2 (Table 2.2). Where positive amplicons in the *Rickettsia*-specific assays were obtained, these were cleaned with ExoSAP-IT kit (E1050, New England Biolabs, US) and Sanger sequenced to validate the amplicon as a true positive, and to allow inference of the relatedness of any strains found. The sequences of *16S rRNA* and *gltA gene* were deposited in the European Nucleotide Archive (ENA) at EMBL-EBI database, the accession numbers are provided in Table 3.3.

3.3.3 Phylogenetic analysis

Sequence chromatograms of *16S rRNA* and *gltA* genes were trimmed of primer sequence and edited in UGENE [258]. All the sequences were exported to fasta format and searched against the NCBI database to find close relatives, as ascertained by BLAST homology. This homology was used to establish if the amplicons were of rickettsial origin. The sequence of markers from closely related strains from other invertebrate hosts were retrieved, and the relatedness within the torix group then estimated. *Rickettsia* strains from other clades were selected to represent the sister group to the torix clade. *Occidentia massiliensis* was used as the outgroup for both topologies. All selected sequences were aligned using the MUSCLE algorithm with its default setting in MEGA X [245, 246]. The Maximum Likelihood

phylogeny for both genes were estimated in MEGA X with 1000 rapid bootstrap replicates under K2+I and T92+G+I model for *16S rRNA* and *gltA* gene respectively

3.3.4 Statistical analysis

It is known that there are taxonomic hot-spots for endosymbiont infection, with for instance spiders being a known hot-spot for a range of microbial symbionts [186-189] I therefore performed analyses that were matched at a taxonomic level (i.e., each taxon was represented in both the aquatic and terrestrial pools). To this end, the incidence of torix *Rickettsia* was first compared in all insects. However, within insects, there is taxonomic heterogeneity between freshwater and terrestrial biomes (e.g., Ephemeroptera, Plecoptera in freshwater only, Lepidoptera in terrestrial only). I therefore focussed the analysis to matching insect orders, present in both the freshwater and terrestrial community. Three insect orders, Hemiptera, Diptera and Coleoptera, fulfilled this criterion with good representation from each biome. For each case, the ratios of 'infected:non-infected' species between freshwater and terrestrial communities were compared in a Fisher's exact test at *p*-value ≤0.05 using the statistical platform R (version 3.6.1, 2019) [259].

3.4 RESULTS

3.4.1 Prevalence of torix *Rickettsia* in invertebrates

Screening of freshwater invertebrates revealed 10 out of 57 species (17.54%) were positive for endosymbiont DNA in PCR assays. The positive species comprised 9 insect species and one mollusc. DNA sequence confirmed that all but one of the infected strains were *Rickettsia* that lie within the torix group (9 of 57 species, 15.79%) (Table 3.3). The final sequence, which was a 16S *rRNA* amplicon from *Corixa punctata*, has a closest BLAST match to a *Trichorickettsia*, a genus within the Rickettsiacae. The list of positive freshwater species is shown in Table 3.4.

For terrestrial invertebrates, PCR assays evidenced *Rickettsia* infection in 10 out of 112 species (8.93%) with a mix of insect and spider hosts (4 and 6 species respectively) (Table 3.3). *Rickettsia* from 8 host species (2 insects and 6 spiders) were identified as lying within the torix clade (8 of 112 species, 7.14%, while the other two host species carried *Rickettsia* from the rhyzobius and bellii group. The list of positive terrestrial species is shown in Table 3.5.

Table 3.3 Summary of screened freshwater vs terrestrial invertebrates. A number in the bracket is the number of all *Rickettsia* positive, while a number in front of the bracket represents only the number of torix *Rickettsia* positive.

	Community	Total species	Infected species	Total individuals	Infected individual	Year range	Geographical range
Invertebrates	Freshwater	57	9(10)	321	13(14)	2002-2017	Central America Europe and UK
	Terrestrial	112	8(10)	1,291	44(54)	2001-2017	Europe and UK

Table 3.4 Freshwater invertebrate species testing positive in the PCR screen where the strains were confirmed by the amplicon sequencing. Number in front of the brackets are the number of infected individuals, while the number inside the brackets indicate the number of tested individuals. Collection, 'a' indicates that the species were collected in this study. The accession numbers for markers are provided where the strains successfully produced the gene amplicons.

	Invertebrate groups and Species	Location	Collection	*N*	Strain	Accession numbers	
						16S rRNA	*gltA*
Trichoptera							
1	*Rhyacophila dorsalis*	Stirling, Scotland, UK	a	2(3)	torix	LR812278	LR812254
Hemiptera							
2	*Corixa punctata*	Ness Gardens, Cheshire, UK	a	1(1)	*Trichorickettsia*	LR961641	N/A
3	*Sigara striata*	Ness Gardens, Cheshire, UK	a	1(2)	torix	LR812279	LR812255
Diptera							
4	*Anopheles plumbeus*	Chester Zoo, Cheshire, UK	a	2(2)	torix	LR813675	LR813676
5	Chironomidae sp.	Ness Gardens, Cheshire, UK	a	1(4)	torix	LR812269	LR812246
6	*Glyptotendipes* sp.	Ness Gardens, Cheshire, UK	a	1(1)	torix	LR812271	LR812248
7	*Hilara* sp.	Sefton park, Merseyside, UK	a	1(3)	torix	LR812272	LR812249
8	*Simulium aureum*	New Forest, Hampshire, UK	a	1(1)	torix	LR812280	LR812256
9	*Zavrelimyia* sp.	Kielder Forest, Northumberlan, UK	a	1(1)	torix	LR812281	LR812257
Gastropoda							
10	*Galba truncatula*	Cheshire, UK	a	3(20)	torix	-	LR812258

Table 3.5 Terrestrial invertebrate species that tested positive in the PCR screen and where the strains were identified by the amplicon sequencing. The number in front of the brackets are the number of infected individuals, while the number inside the brackets indicate the number of tested individuals. Collection, 'a' indicates that the species were collected in this study, 'b' indicates the species that were obtained in DNA extracts from Duron *et al*. [188]. Accession numbers are provided when the strains successfully produced gene amplicons.

	Invertebrate groups and Species	Location	Collection	*N*	Strain	Accession numbers	
						16S rRNA	*gltA*
Araneae							
1	*Amaurobius fenestralis*	Montpellier, France	b	1(6)	torix	LR899445	LR961638
2	*Hylyphantes graminicola*	Ness Gardens, Cheshire, UK	a	1(1)	torix	N/A	LR961639
3	*Linyphia triangularis*	Berlin, Germany	b	9(9)	torix	LR812273	LR812250
4	*Pholcus phalangoides*	Berlin, Germany	b	17(20)	torix	LR812275	LR812251
5	*Pisaura mirabilis*	Greater London, UK	b	1(12)	torix	N/A	LR812252
6	*Pardosa lugubris*	Darmstadt, Germany	b	1(20)	torix	N/A	LR961640
Hemiptera							
7	*Cimex lectularius*	Yorkshire, UK	b	12(12)	torix	LR828195	LR828196
Coleoptera							
8	*Phyllobius argentatus*	Mont Barri, France	b	4(15)	rhizobius	LR812276	N/A
Diptera							
9	*Protocalliphora azurea*	Montpellier, France	b	12(12)	torix	LR812277	LR812253
Hymenoptera							
10	*Pachycrepoideus* sp.	UK	b	6(94)	bellii	LR812274	-

3.4.2 Freshwater vs terrestrial hosts

To reduce taxonomic 'hot-spot' biases, I first compared the incidence of *Rickettsia* infection in aquatic vs terrestrial insects. Fisher's exact test analysis rejected the null hypothesis of equal representation, with freshwater taxa having a higher representation of species with torix *Rickettsia* than terrestrial (*p*-value = 0.013, infection in 8 of 47 freshwater versus 2 of 75 terrestrial species respectively, Figure 3.1 A).

Examining the narrower phylogenetically controlled set (the three matched insect orders), revealed the infection of torix *Rickettsia* in 7 of 31 freshwater (22.6%) and 2 of 53 terrestrial insects (3.8%) (Table 3.6). Fisher's exact test analysis again rejected the null hypothesis of equal representation with freshwater taxa again having a higher representation of species with torix *Rickettsia* than terrestrial (Fisher's exact test, *p*-value = 0.025, Figure 3.1 B).

Table 3.6 Summary of screened freshwater vs terrestrial invertebrates from the three focussed insect orders. A number in front of the brackets represents the number of torix *Rickettsia* positive. A number in the bracket is the number of total screened species/individuals.

Insect Order	Community	No. species	No. individual	Year range	Geographical range
Hemiptera	Freshwater	1(9)	2(55)	2006-2016	Europe and UK
	Terrestrial	1(11)	12(142)	2006-2008	Europe and UK
Diptera	Freshwater	6(18)	7(150)	2002-2017	Central America, Europe and UK
	Terrestrial	1(16)	12(156)	2001-2017	Europe and UK
Coleoptera	Freshwater	0(4)	0(20)	2006-2017	Europe and UK
	Terrestrial	0(20)	0(279)	2004-2016	Europe and UK
Total insects	Freshwater	7(31)	9(225)		
	Terrestrial	2(47)	24(577)		

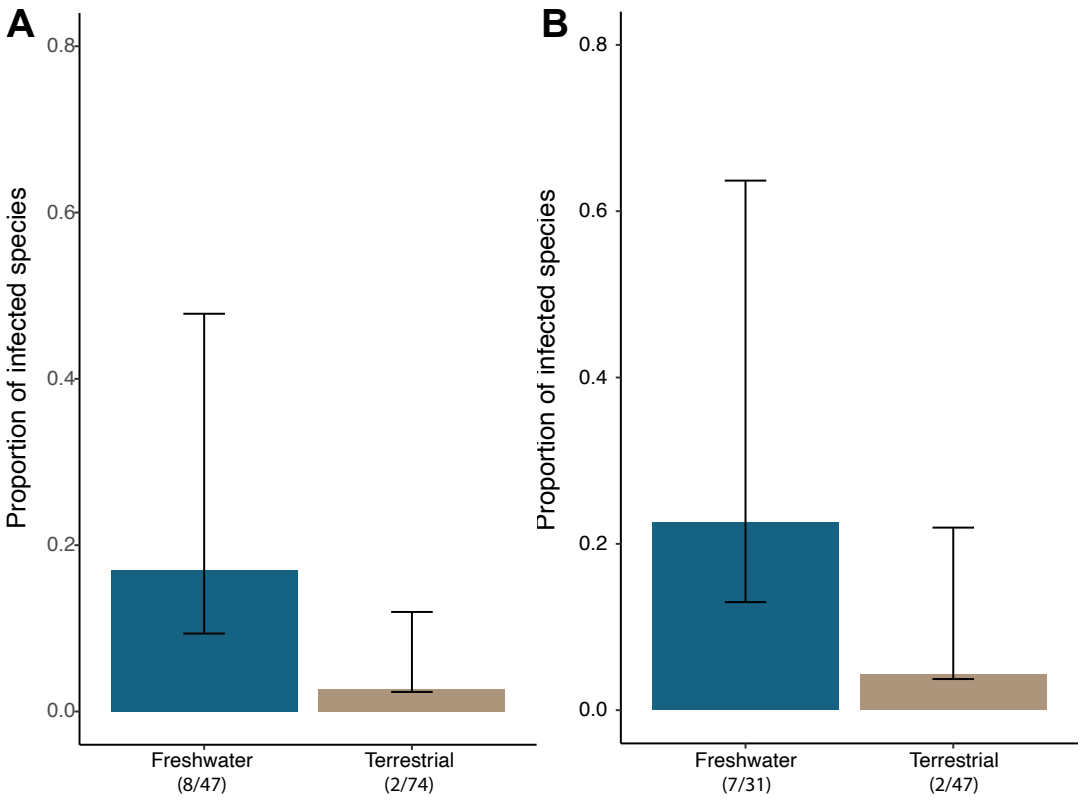

Figure 3.1 Prevalence of torix *Rickettsia* in freshwater and terrestrial invertebrates. **A**: Comparisons between freshwater and terrestrial species from all insect orders. Fisher's exact test revealed a significant difference between the proportion of infected species from the two types of host communities (*p*-value = 0.013). **B:** Comparisons between freshwater and terrestrial species from three insect orders; Hemiptera, Diptera and Coleoptera. The proportion of infected species was significantly different between the two host groups (Fisher's exact test; *p-value* = 0.025). Error bars represent 95% confidence intervals derived from binomial sampling.

3.4.3 The relatedness of *Rickettsia*

There were 15 *Rickettsi*a strains from 19 infected host invertebrates (8 from freshwater and 7 from terrestrial community) that successfully produced 16S amplicons and retrieved sequences. Reconstruction of *16S rRNA* phylogeny indicated that the strains were mostly placed in torix group *Rickettsia* with a mix of freshwater and terrestrial invertebrates. Two terrestrial insect hosts, the short-nosed weevil (*Phyllobius argentatus*) and the parasitoid wasp (*Pachycrepoideus* sp.) were affiliated in rhyzobius and bellii clades, respectively. (Figure 3.2).

The topology for the *gltA gene* phylogeny revealed 17 strains (9 from freshwater and 8 from terrestrial groups) allocated to the torix group *Rickettsia*. The strains that were affiliated to the rhyzobius and bellii clades, according to *16S rRNA* topology, failed to produce *gltA* amplicons (Figure 3.3).

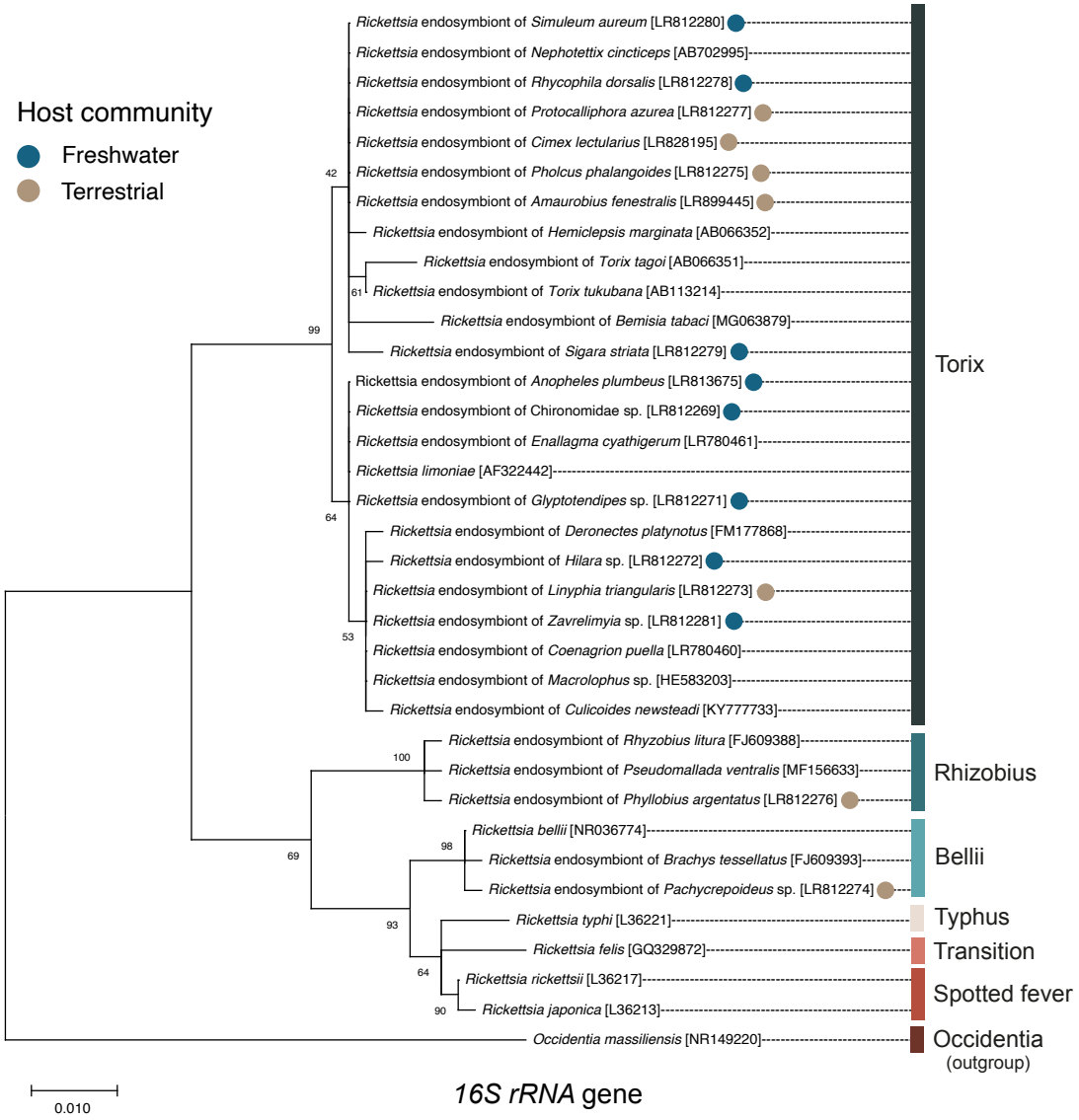

Figure 3.2 ML phylogenetic tree based on *16S rRNA* gene. The topology is reconstructed with K2+I model in MEGA X. The strains found in this study are marked with coloured circles. The majority of *Rickettsia* endosymbionts from this study are in torix group. *Rickettsia* strains found in *Phyllobius argentatus* and *Pachycrepoideus* sp. are affiliated with rhizobius and bellii clades, respectively. Numbers on the nodes indicate bootstrap value of 1000 replicates. Scale bar represents the rate of nucleotide substitution per site.

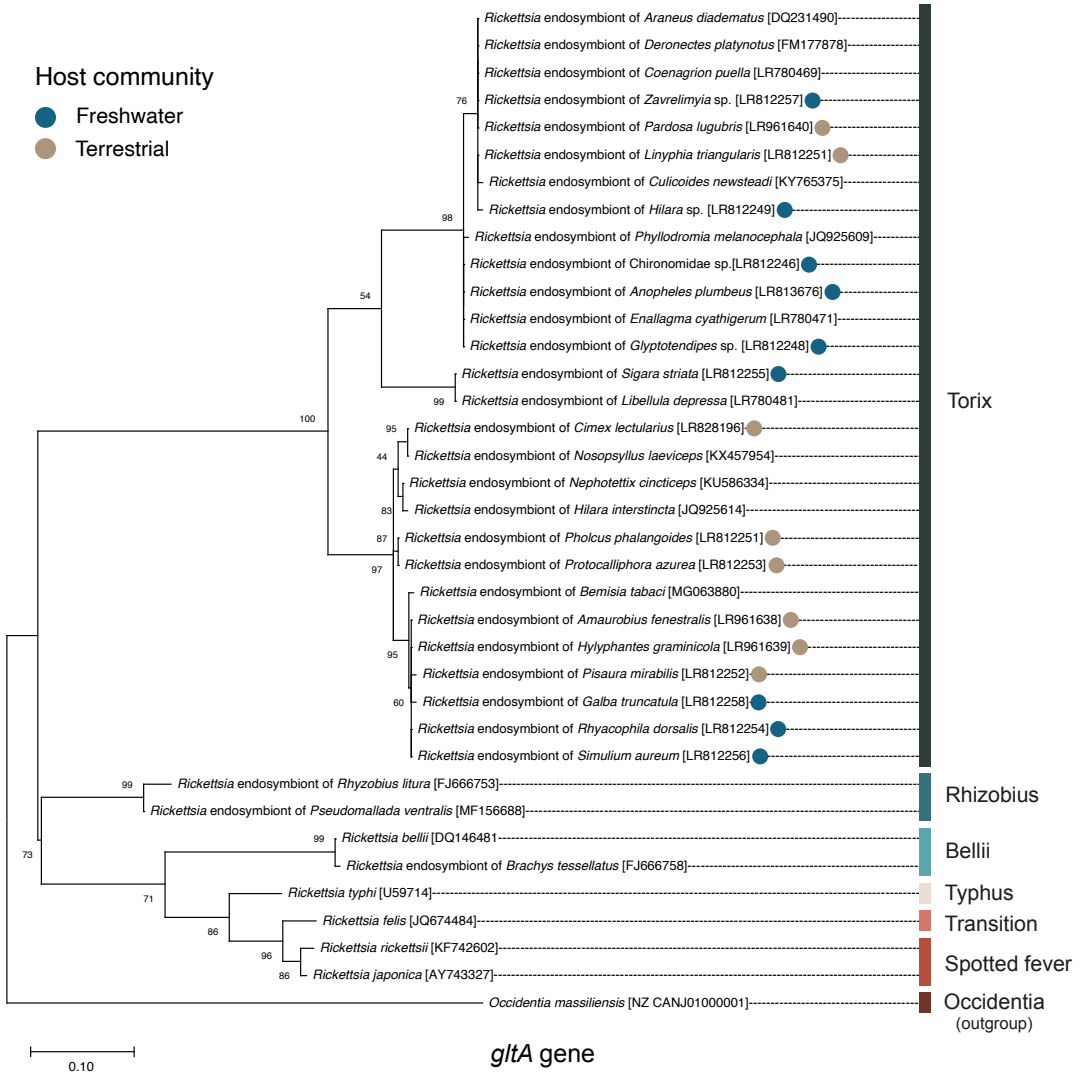

Figure 3.3 ML phylogenetic tree based on *gltA* gene. The topology is reconstructed with T92+G+I model as the best nucleotide substitution model, implemented in MEGA X. The strains found in this study were marked with coloured circles. All of *Rickettsia* endosymbionts that were amplified from *gltA* primers are affiliated with torix group. Numbers on the nodes indicate bootstrap value of 1000 replicates. Scale bar represents the rate of nucleotide substitution per site.

3.5 DISCUSSION

Recent studies have discovered many invertebrate-associated *Rickettsia*. Many of the recently published *Rickettsia* sequences are affiliated in 'torix' clade, which originated from the finding of *Rickettsia* in the leech *Torix targoi* [9]. Other related strains within this clade derive from other leech species [135], amoeba [164], spiders [260], deronectid beetles [169], biting midges [175] and odonates [197]. Weinert *et al*. in 2009 [182] revealed the diversity of *Rickettsia*, and delineated 12 groups, including the torix clade. They hypothesized that 'torix *Rickettsia* are more abundant in freshwater ecosystems' based on the infection evidence of several freshwater organisms. The records since this date are consistent with this hypothesis [175, 193, 197, 256, 257], but a formal unbiased tested has not been made.

This study surveyed torix *Rickettsia* infection in invertebrate hosts from two major communities, freshwater and terrestrial ecosystems, to test this hypothesis. I compared the proportion of infected species between the two host communities. The results revealed first that torix *Rickettsia* were found widely in taxa within the aquatic biome, adding Gastropoda to the list of taxa carrying the symbiont infection. Analysing samples matched for taxonomy supported the hypothesis that torix *Rickettsia* were more abundant in freshwater hosts than terrestrial taxa.

3.5.1 Diversity of torix *Rickettsia*

The majority of *Rickettsia* endosymbionts found in this study are affiliated with the torix clade. This clade comprises the strains from other invertebrates, e.g., leeches (*Torix targoi*, *T. tukubana* and *Hemiclepsis marginata*), a biting midge (*Culicoides newstedi*), a diving beetle (*Deronectes platynotus*), a leaf hopper (*Nephotettix cincticeps*), a white fly (*Bemisia tabaci*), a spider (*Araneus diadematus*), a human flea (*Nosopsyllus laeviceps*) and odonates (*Coenagrion puella*, *Enallagma cyathigerum* and *Libellula depressa*), etc (Figure 3.2 and 3.3). It could be seen that all *Rickettsia* strains were retrieved from a mix of freshwater and terrestrial invertebrate hosts.

This close relationship between the torix *Rickettsia* from both host communities (albeit based on two marker sequences) indicates the movement of *Rickettsia* across invertebrate species and between biomes (Figure 3.2 and 3.3). For instances, *Rickettsia* endosymbiont of a caddis fly, *Rhyacophila dorsalis,* are clustered in the same group of the strains found in spiders (e.g., *Amaurobius fenestralis* and *Pardorsa lugubris*) and a common bed bug (*Cimex lectularius*). These symbionts seem to have a close relation to each other while their hosts are phylogenetically distant. This movement naturally occurs when the host acquires symbionts through horizontal transmission events [261, 262]. Many symbionts are horizontally transferred over an evolutionary timescale. This process expands both the number and range of host species [263]. Direct agents for the horizontal transfer may involve parasitism events (e.g., parasitic wasps, twisted wings, and water mites) [262, 264-266]. Acquisition may also occur via the food chain when the invertebrates feed and digest their food plants or prey [154, 262, 267, 268].

3.5.2 The driving pattern of torix *Rickettsia* infection

This study indicates that freshwater ecosystems represent a hot-spot for torix group *Rickettsia*. This includes semi-aquatic invertebrates where only a part of their lifecycle is in the aquatic realm, e.g., crane fly and biting midge. How this pattern arises is uncertain. One possibility is that water itself is the medium enabling horizontal transmission to happen. A second possibility is that microeukaryotes within the water may be a common source – for instance amoebae [164, 269]. This horizontal transmission within the protozoans was found in '*Candidatus* Megaira', a relative of the genus [270]. Finally, infection might be horizontally passed through other freshwater species via the food web. Freshwater hosts with a terrestrial phase (e.g., caddis flies, midges, odonates) may also act as a bridge to the terrestrial community.

3.5.3 Freshwater vs terrestrial, sampling bias between communities

The data revealed that insect species from the aquatic community were more likely to carry *Rickettsia* than ones from the terrestrial community. It is important to determine whether this result could be a result of sampling bias rather than an actual

difference. It is known that symbiont infections can reside in a fraction of the population, and thus increased sampling would likely elevate the chance of finding a symbiont infection in a focal species. However, the results indicating evidence of torix *Rickettsia* abundance in freshwater biome were conservative on this criterion. Based on the aspect of sampling intensity, generally, most invertebrate species from freshwater biome were sampled with lower numbers when compare to the sample sizes of those from terrestrial habitats. For instance, the mean sample size of freshwater hemipterans, dipterans and coleopterans were 6.1, 8.34 and 5.0 individuals/species respectively, while terrestrial hemipterans, dipterans and coleopterans were sampled at mean intensity 12.9, 9.8 and 13.3 individuals/species respectively. From this, it could be said that sampling alone would have biased records of *Rickettsia*-infected individuals to species from the land and make it less likely to detect the infection in freshwater samples as there were fewer numbers of individual samples per species. Thus, the true *Rickettsia* incidence in freshwater communities may be even higher than the results reported in this study, and the excess of *Rickettsia* associations in aquatic compared to terrestrial species more pronounced than observed here.

3.5.4 Detection of torix *Rickettsia* from contaminated gut contents

In this study, all DNA templates were retrieved from either the whole body or dissected abdomen of the invertebrates. There might be a chance to detect torix *Rickettsia* from contaminated digestive tract contents (e.g., small insect foods or microeukaryotes [164, 269]) instead of the host tissue-associated torix *Rickettsia*, which may lead to a mis-interpretation of an infection incidence. In order to reduce such a risk, a few criteria were also considered, to validate the endosymbionts. First, to ensure that the DNA templates were retrieved from the actual host species, rather than a mix of various host organism in the gut, the DNA sequences from COI gene were checked. Second, the brightness of *Rickettsia* amplicons on the gel electrophoresis were visually determined. The brightness of the amplicons likely reflected that the infection titres were high enough to be retrieved within the host tissue. Then, the chromatogram of *gltA* and *16S rRNA* gene sequences were also checked for quality. However, although these seemed to help reduce the risk, they

still allowed the chance of mis-detection to occur. Alternatively, an investigation by selecting least contaminated tissues (e.g., legs or upper parts of the body) and increasing a number of samples/species would be more helpful to ensure that the detection of the endosymbiont is from the actual host species.

3.5.5 Torix *Rickettsia* a symbiont of vector and blood-feeder invertebrates

Some of the torix *Rickettsia* found in this study (Figure 3.2 and 3.3) noted a few host species of the symbionts that are haematophagous ectoparasites of vertebrates and a potential vector of trematodes and nematodes.

A freshwater snail (*Galba truncatula*), that hosts for torix *Rickettsia* in this study, has been reported as an intermediate host for a variety of trematodes and nematodes [271-273]. The infection of torix *Rickettsia* was likely genuine in this snail population, as a contamination of *Rickettsia* in their foods (i.e., algae) was not detected (from a personal observation). However, the prevalence of *Rickettsia* infection in this snail (Table 3.4) - three of twenty specimens were positive in the PCR assays - indicates low level of infection frequency in this laboratory populations. An exploration through a larger sample size might help reveal a segregation of torix *Rickettsia* in this vector species.

The DNA sequences retrieved from Genbank consist of *Rickettsia* from a mix of freshwater and terrestrial blood-feeding invertebrates. Glossiphoniid leeches (*T. targoi, T. tukubana* and *H. marginata*) are in freshwater ecosystems feeding on fish, amphibians, reptiles and mammals [9]. Biting midge (*Culicoides newsteadi*), a semi-aquatic species and gerbil flea (*Nosopsyllus laeviceps*), a terrestrial parasite, both feed on mammals and birds [274, 275]. Similarly, the strains found in this study are a mix of both communities. The black fly (*Simulium aureum*) is a lotic species [276]. The mosquito (*Anopheles plumbeus*) is freshwater species with adults that feed on humans and birds [277]. Finally, a human bed bug (*Cimex lectularius*) was infected, a terrestrial specialist ectoparasite whose diet is restricted to the blood of humans [278]. These blood-feeding associated *Rickettsia* seem to have significant impact in medical areas, especially the latter two insect species.

Although torix *Rickettsia* could be found in several blood-feeding invertebrates, there was no statistical evidence to support that this endosymbiont is particularly prevalent in these animals. I, therefore, further examined ratios between number of torix *Rickettsia* infection in blood-feeding dipteran species vs non blood-feeding dipterans. The comparisons indicated no statistical significance between the two ratios (Fisher's exact test, *p*-value = 0.584, torix *Rickettsia* infection in blood-feeding dipterans = 2/7, in non-blood-feeding dipterans = 4/26).

However, *Anopheles plumbeus* and *C. lectularius* are good candidate models to observe the influences of torix *Rickettsia* on these insect vectors as they have the potential to be established in laboratory systems. The leeches and biting midge associated *Rickettsia* have already had their biological aspects investigated, including the vectors-symbiont interactions [135, 175]. Further intensive study on torix *Rickettsia* in either of these two insect vectors will help to understand the roles of endosymbiont in aspects of host-symbiont biology.

3.5.6 Non torix *Rickettsia*

Apart for the detection of torix *Rickettsia* in this study, other *Rickettsia* strains, i.e., rhyzobius and bellii are also observed (Figure 3.2). The bellii strain was detected from the parasitioid wasp *Pachycrepoides* sp., which is related to a strain that was observed to cause thelytokous parthenogenesis in another parasitiod wasp species (*Pnigalio soemius*) [90]. Moreover, *Rickettsia* in this group was also found in a whitefly (*Bemesia tabaci*), the host species that were recently reported as another host for torix *Rickettsia* in the study of Wang *et al*. in 2020 [257]. This latter study also suggested evidence of horizontal movement of the symbiont via the plant-mediated system.

Rickettsia endosymbiont of a weevil (*Phyllobius argentatus*) is grouped in rhyzobius clade (Figure 3.2). This clade was also reported in other beetles and known to be common in Neuroptera (lacewings) [181]. However, the biological impact from this group has yet to be observed, except the suggestion of strict vertical transmission from the lacewing's ancestral species.

Finally, the allied bacterial group *Trichorickettsia* was detected in a lesser water boatman (*Corixa punctata*) under the PCR assays. The BLAST analysis revealed the closest related strain was *Candidatus* Trichorickettsia mobilis, a cytoplasmic intracellular bacterium of *Paramecium nephridiatum* [279]. The bacterium is a member of order Rickettsiales (Alphaproteobacteria), which this genus is believed to be the sister clade of *Rickettsia* genera [280] and commonly associates with freshwater protist ciliates [281]. The detection of *Trichorickettsia* suggests the association may not exist within the insect tissue, but the insect host may already have accidentally acquired the ciliate hosts inside their digestive tracts. The discovering of *Trichorickettsia* in this study also implies the broad range amplifiability of the 16S rRNA primers. The variable site of this pair and the priming site on *16S* region of *Trichorickettsia* may need to be revised, even though this primer set has been validated in the previous studies and confirmed the specificity to *16S rRNA* region of *Rickettsi*a, not other genera in Rickettsiales [169, 175].

3.6 CONCLUSION

It has been hypothesised that torix *Rickettsia* are generally associated with freshwater organisms. This study revealed the first statistical evidence of the torix *Rickettsia* abundance in the freshwater biome. Although the comparisons were based on closely phylogenetical taxa within only several insect orders, the sampling bias towards terrestrial individuals/species suggests the estimate conservative. Most of the detected symbion*t* strains in this study were identified as torix *Rickettsia,* which were highly diverse across freshwater and terrestrial host species. This study also highlighted that torix *Rickettsia* is a common endosymbiont of haematophagous invertebrates, e.g., *Anopheles plumbeus* and *Cimex lectularius*, which the vector hosts have potential important role in disease. Therefore, in the next Chapter, I will perform an investigation with more intensive examination of the symbiosis between the common bed bug, *C. lectularius*, and torix *Rickettsia* and establish this as the host-symbiont system for more in-depth studies in future.

CHAPTER 4

Torix *Rickettsia*: a maternally inherited endosymbiont in the common bed bugs (*Cimex lectularius*)

Publication and author contributions

All results in this chapter, except FISH imaging (Figure 4.8), is in press at *Frontiers in Microbiology* with a bioRxiv pre-print under Thongprem *et al.* in 2020 [282]. All *Cimex lectularius* materials were obtained from Dr Oliver Otti, University of Bayreuth, Germany, and Dr Sophie Evison, University of Nottingham. Bed bug crossing in transmission mode experiment was conducted by Dr Oliver Otti and Dr Sophie Evison. DNA extraction, PCR screening, Sanger sequencing, FISH imaging and all data analysis were completed by me with advice from my supervisor, Prof Greg Hurst.

4.1 ABSTRACT

The common bed bug, *Cimex lectularius,* is a human pest that has globally infested domestic habitats, and is known to be symbiosed by the obligative α-proteobacterium (*Wolbachia)* and a facultative γ-proteobacterium (BEV-like symbiont). The previous chapter highlighted the presence of *Rickettsia* as an additional, previously unrecognised, facultative alpha-proteobacterial symbiont of this species. However, the biological aspects of the interaction between the bug and *Rickettsia* have not yet been determined. In this study, I first revealed that *Rickettsia* is likely common in *C. lectularius,* present in 13 of 21 lab populations (61.9%) originally collected from three geographical areas, Africa, Europe and Great Britain. The phylogenetic trees based on *16S rRNA* and *gltA* gene sequences illustrated that these strains are identical and affiliated with the torix clade. Crossing studies were completed and indicated the *Rickettsia* is maintained in *C. lectularius* generations via maternal but not paternal passage. Fluorescence *in-situ* hybridisation in the infected bed bugs showed infection in ovaries and other somatic tissues, and the presence of *Rickettsia* signals in bacteriomes where *Wolbachia* and BEV-like symbionts are also located. Being vertically inherited, bed bug-associated *Rickettsia* influences on *C. lectularius* biology merit ongoing investigation.

4.2 INTRODUCTION

4.2.1 General introduction to bed bug biology

The common bed bug (*Cimex lectularius*) is a wingless hematophagous insect belonging to order Hemiptera, family Cimicidae. The insects in this family feed on the blood of many vertebrates including humans [278, 283, 284]. Bed bugs are common in temperate regions, and have re-infested globally over recent decades [285, 286] (more details for each region in Doggett *et al*. [287]). They are an important pest, as they have significant medical, social and economic impacts [288-291]. Infestation with *C. lectularius* is mainly a discomfort to human lifestyle, as the species has not yet been observed to be a natural vector of any arthropod-borne diseases [291-295].

Bed bugs are dorsoventrally flattened, oval shape and reddish-brown to light brown colour. The adults are 4.0-5.0 mm long and 1.5-3.0 mm wide [285, 296]. Females are bigger than males in general and have more rounded abdomen (Figure 4.1). Like the other hemimetabolous hemipterans, bed bugs develop from nymph to adult with incomplete metamorphosis [297]. There are five nymphal stages before entering into the adulthood. In each stage they take 5-8 days to molt into the next stadium, at least one sufficient blood intake is enough for each subsequent molting [298, 299]. Typically, the life cycle (egg to egg) takes 5 weeks at 75-80% RH and 28-32^{o}C [285]. Female adults lay whitish fertile eggs (1 mm long) in clusters. Adult bed bugs may live for 1-2 years [292, 300], but will be less if they live in unsuitable conditions [301] and when blood meals are inaccessible [285, 299]. Bed bugs are naturally nocturnal but may feed in daytime if they are hungry. The feeding frequency is varied depending on their digestion rate, surrounding temperature, host availability and reproductive cycle [299, 302].

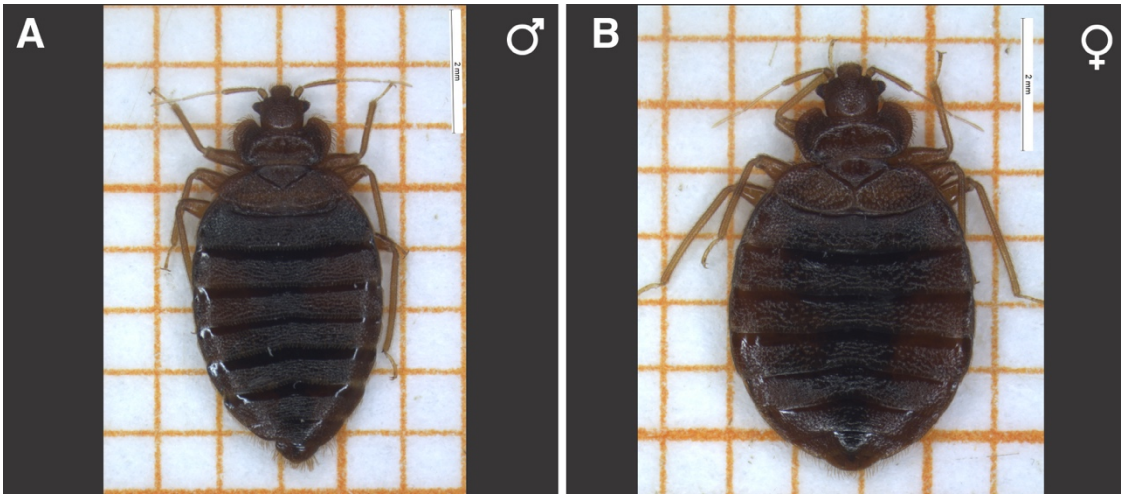

Figure 4.1 Pictures of the common bed bug (*C. lectularius*). Dorsal view of adult male (A) and female (B) on 1 mm grids.

The blood meal stimulates the reproductive system of the adults to be ready for copulation, egg production in females and sperm production in males [303]. The mating system of *C. lectularius* and its allies is unique, known as 'traumatic insemination'. Rather than inserting the stylus-liked genital organ (paramere) into female genitalia, the bed bug male intromittent organ will insert right into female abdominal sternite at a suture area (Figure 4.2 A and B) through the body wall where the special sperm receiving organ called 'mesospermalege' is located underneath. This mating event is believed to activate ovarian development in females (Figure 4.2) [303]. The injected sperm will leave mesospermalege and migrate via haemocoel to sperm repository organ (seminal conceptacle) using oxygen gradients in haemolymph [304]. When ovarian development is completed, the sperm will be moved to ovaries (Figure 4.2), where fertilization takes place.

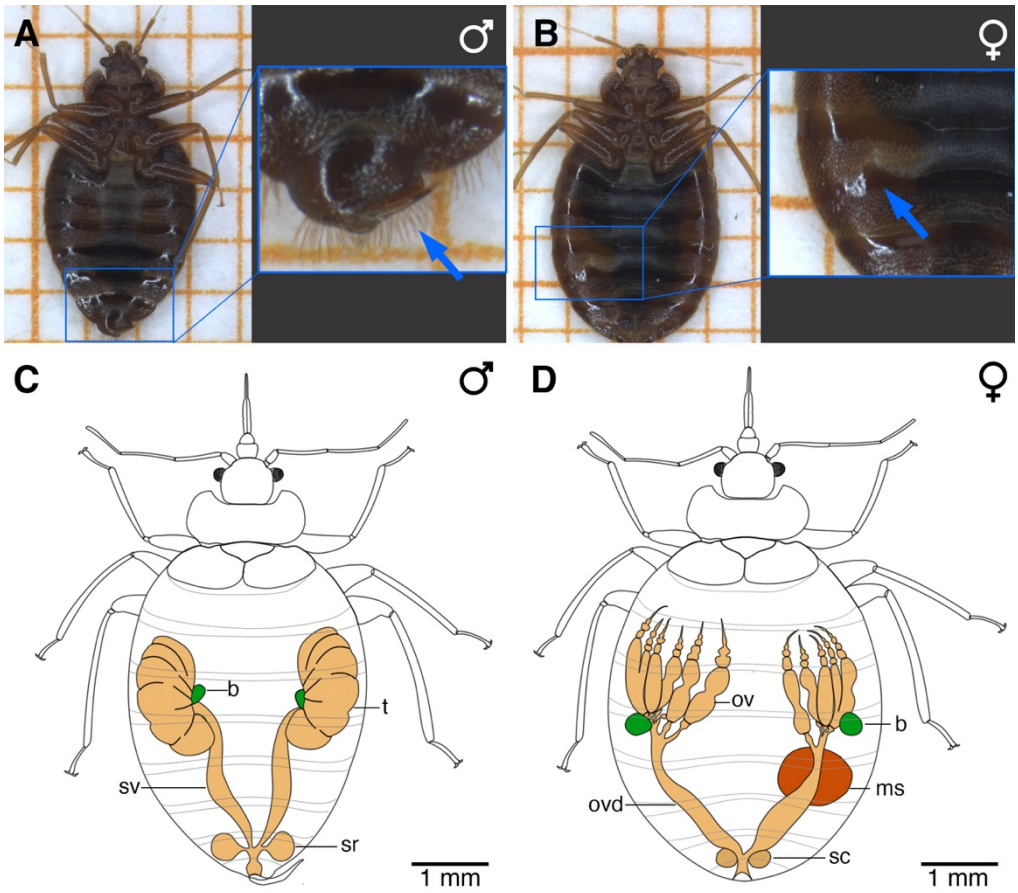

Figure 4.2 Reproductive organs of the common bed bug (*C. lectularius*). **A**: The intromittent organs of male. **B**: The abdominal sternite suture of female where mesospermalege (ms) is allocated underneath the exoskeleton. **C** & **D**: The illustrations show reproductive organs and bacteriomes (b) allocations in male and female. ov = ovary, ovd = oviduct, sc = sperm conceptacle, sr = sperm reservoir, sv = seminal vesicle, t = testis.

4.2.2 Diversity of Cimicidae and human bed bugs

All the member of insects in this family are obligate blood feeders and live as natural ectoparasite of mostly warm-blooded animals, e.g., bats and birds [305, 306]. There are approximately 110 described cimicid species [283] from 6 subfamilies [185, 307], i.e., Primicimicinae, Latrocimicinae, Cimicinae, Cacodminae, Haematosiphoninae and Afrocimicinae. Three species are considered as specialist human ectoparasites. *Leptocimex boueti*, a member of subfamily Cacodminae, is associated with people in South Africa [308]. The other two species are in subfamily Cimicinae, the common bed bug (*C. lectularius*) and its cousin tropical bed bug (*C. hemipterus*), which infests many countries in temperate and equatorial areas, respectively [278, 307].

4.2.3 *Wolbachia* as the primary endosymbiont

Bed bugs are obligatory blood feeders over their entire life history, a diet which is considered depauperate in B vitamins. This narrowed food preference of the ectoparasitic niche requires other living organisms to supplement their insufficient nourishment which they can neither produce nor obtain from their host. Many cimicid species associate with *Wolbachia* endosymbionts [309-314], and these have been found to synthesize Biotin, an inaccessible B-vitamin compound [6, 315]. This endosymbiont has a mutualistic relationship with the bug, in which the bug provides a special organ called a 'bacteriome' for harbouring them [6, 316].

Bed bug associated *Wolbachia* belongs in the F supergroup. The strain is a close relation to A, B and D supergroups found in other insects and nematodes associations [314, 317]. The distinctiveness that makes this strain differ from others is that the genome contains complete pathways encoding biotin and riboflavin synthesis, the major components of vitamin B_7 and B_{12}, synthetic pathways [2, 315]. Although the titre of these symbionts is dynamic in *C. lectularius* populations and each instar stadium [318], bed bugs that are dissociated with the bacteria (cured with rifampicin antibiotics) will no longer maintain their biological fitness, e.g., decreasing in fecundity and slowing in development, unless the diet is artificially supplemented with vitamins [6].

4.2.4 BEV-like symbiont and *Rickettsia*, the facultative endosymbionts

Besides harbouring *Wolbachia* as the primary endosymbionts, *C. lectularius* also form facultative interactions with a second bacterium. A γ-proteobacterium was first described at the same time as *Wolbachia* by Hypša and Aksoy [311] from ovaries of *C. lectularius.* This rod-shaped bacterium is originally identified and cultivated from leafhopper, *Euscelidius variegatus*, also known as 'BEV' (the bacterium of *E. variegatus*) [319-321]. The BEV-like symbiont is likely common in insects in the order Hemiptera [6, 311, 320]. Despite the fact that *Wolbachia* and BEV-like symbiont are the major endosymbiont groups in many bed bug populations, a study from more than 90 years ago already revealed the first incidence of *Rickettsia,* another endosymbiont, as a potential parasitic endosymbiont associated with bed bugs. *Rickettsia*, described by Arkwright *et al*. in 1921 [322], were found in cells of mesospermalege and Malpighian tubes under a light microscope. The study described two form of *Rickettsia*, motile thread-like and non-motile coccus forms, thought the motile form seems to be BEV-like symbiont in other studies [6, 319-321]. A few later studies noted the presence of *Rickettsia* in *C. lectularius* but haven't highlighted the endosymbiont biology of this interaction [323, 324].

More recently, Potts *et al*. [325] observed *Rickettsia* associated with bed bugs as an endosymbiont from natural populations in UK and USA using PCR assays with rickettsial specific primers based on citrate synthase gene (*gltA*) [326]. The genetic evidence showed that this strain is closely related to the *Rickettsia* found in a gerbil flea (*Nosopsyllus laeviceps*) [183]. However, the study did not establish the biology of the endosymbiosis and the interactions between the bacterium and the host.

4.2.5 Incidence of *Rickettsia* in *C. lectularius* in this study

The *Rickettsia* screen in Chapter 3 identified, *Rickettsia* in all twenty *C. lectularius* DNA samples. The bed bug samples (population name 'F4') were originally collected from London and established in the laboratory in University of Sheffield in 2006 (see Table 4.1 in the method). Then, in 2018, I explored more individuals from the same Sheffield laboratory stock, and infection was observed to remain but be sporadic within the population (mixed infected/uninfected individuals). This mixed

infection led to the idea of establishing *Rickettsia* infected and *Rickettsia* uninfected bed bugs lineages to observe the biology of *Rickettsia* endosymbiont and *Rickettsia*-bed bug interactions, a scenario that could not be completed using antibiotics, as these would eliminate *Wolbachia* that are vital for *C. lectularius.*

4.2.6 Aims

Bed bugs represent a good subject to establish a model system of *Rickettsia*-host interactions as they are easily maintained in lab conditions and their life cycle is short. As noted in Chapter 3, *C. lectularius* are potentially associated with *Rickettsia* endosymbiont, and this leads to the first aim, to examine the infection prevalence over cosmopolitan populations. Following this, the presence of infected and uninfected isolines of *Rickettsia* provides a good opportunity to study and understand the general biology of bed bug-associated *Rickettsia.*

In this chapter, I undertook PCR assays and Sanger sequencing to observe the prevalence and diversity of torix *Rickettsia* across *C. lectularius* populations and other cimicid species. This would allow me to understand the relationship of the torix *Rickettsia* strains from this study and other *Rickettsia* strains from different invertebrate host species. I also investigated the transmission pattern of the symbionts, with a particular view of establishing whether paternal transmission – observed in another torix *Rickettsia*-insect symbiosis [249]– is also observed in bed bugs. Finally, I localised the infection of this symbiont in *C. lectularius* tissues. These observations of torix *Rickettsia* biology will further be useful to acknowledge and predict the biological effects on the host biology.

4.3 METHODS

4.3.1 Bed bug populations and other Cimicidae collections

Bed bug populations used in this study were collected in different areas and different years in Europe and Africa, and then maintained in the laboratory (Table 4.1). Two populations are of unknown origin in the wild. Population S1 has been maintained at the Universities of Bayreuth and Sheffield for >20 years and before that for >40 years at the London School of Hygiene and Tropical Medicine. The other population of unknown origin were received from Bayer labs (Germany) in 2006 and have been in the lab ever since.

DNA template of various cimicid species were obtained from the recently published bed bug phylogeny by Roth *et al*. [305] (Table 4.2). For each species, the DNA template was acquired as 10 µl extracted genomic DNA in 0.2 µl tubes from Steffen Roth (University Museum of Bergen, Norway) and Prof. Klaus Reinhardt (TU Dresden, Germany), which were stored in -20^{o}C until use. Voucher specimens of some species are stored in the collection of the University Museum of Bergen (ZMNB), Norway.

4.3.2 Genomic DNA extraction

A pair of male/female of *C. lectularius* from each population (Table 4.1) were sent in absolute ethanol tubes to the lab in University of Liverpool for DNA extraction. The samples were rinsed with clean absolute ethanol and air dried. To avoid contamination with gut microbes, the bugs were decapitated with sterilized forceps and only the head and/or the upper part (from the head to the thorax, including legs) would be taken for DNA extraction. Genomic DNA was extracted from the selected body part using Promega Wizard® Genomic DNA Purification kit (A1120, Promega, UK), adapted from the manufacturer protocol (see section 2.3.1 in Chapter 2). DNA pellets were dissolved with 100 ul of molecular water and stored at -20^{o}C for future use.

Table 4.1 *Cimex lectularius* laboratory populations. A male and female/population were sent for *Rickettsia* screening. Except for Bayer and S1, all populations originated from independent infestations in private homes or youth hostels (Populations H1 and YMCA). The time when they were established in the lab in University of Sheffield are shown in year.

Population name	Origin of place	Year established in Sheffield
H1	Budapest, Hungary	2010
C1	Coventry, UK	2010
Bayer	Germany	2006
S1	London School of Tropical Medicine	1996
London heavy	London, UK	2006
F11	London, UK	2006
F4	London, UK	2006
F10	London, UK	2006
YMCA	London, UK	2010
K12	Nairobi, Kenya	2008
K4	Nairobi, Kenya	2008
K22	Nairobi, Kenya	2010
K25	Nairobi, Kenya	2010
K26	Nairobi, Kenya	2010
K19	Nairobi, Kenya	2010
K20	Nairobi, Kenya	2010
K7	Nairobi, Kenya	2008
K5	Nairobi, Kenya	2008
K30	Nairobi, Kenya	2010
BG1	Sofia, Bulgaria	2010
K17	Watamu, Kenya	2010

Table 4.2 The cimicid allies of *C. lectularius*. This Cimicidae collection was either collected from wild or curated in a museum, compiled by Robert Leslie Usinger (U) and privet donors (P), the Essig Museum of Entomology, University of California, Burkeley. The specimens were originally collected from different localities and times.

Subfamily	Species	*N*	Source	Locality	Year of collection
Afrocimicinae	*Afrocimex constrictus*	3	wild[P]	Kenya	2005
Primicinae	*Bucimex chilensis*	1	museum[U]	Chile	2013
	Primicimex cavernis	1	museum[U]	Mexico	2015
Haematosipho-ninae	*Ornithocoris pallidus*	1	N/A	USA, South Carolina	2010
	Acanthocrios furnarii	1	N/A	Brazil	2010
	Psitticimex uritui	1	N/A	Argentina	2008
	Cyanolicimex patagonicus	1	N/A	Argentina	2003
	Cimexopis nyctalis	1	N/A	USA	2016
	Hesperocimex sonorensis	1	N/A	Mexico	2017
	H. coloradensis	1	museum[P]	Los Alamos County, N.Mex.	1971
Cimicinae	*Paracimex inflatus*	1	museum[U]	Kavieng, Papua New Guinea	1966
	P. borneensis	1	N/A	Borneo	2015
	Cimex pipistrelli	1	N/A	Hanau, Germany	2004
	C. hemipterus	1	museum[U]	Taiwan	Before 1966
	C. hirundinis	1	N/A	Switzerland	N/A
Cacodminae	*Aphrania elongata*	1	N/A	Senegal	2012
	A. vishnou	1	museum[U]	Phnom Penh, Cambodia	1952
	Cacodmus villosus	1	N/A	Kenya	2005
	Loxapsis malayensis	1	museum[U]	Tasik Bera, Pahang, Malaysia	1962
	Leptocimex duplicatus	1	N/A	Israel	2002
	L. boueti	1	N/A	N/A	N/A

4.3.3 *Rickettsia* screening across *C. lectularius* lab populations and other Cimicidae

The DNA samples were initially tested for their amplifiable quality, using the combination of invertebrate mtDNA barcoding primers, forward; C1J_1718 and reverse; HCO_2198 to amplify approximately 380 bp product size in the Cytochrome oxidase subunit I gene (*COI)* of the bed bug using PCR. The primer sequences and the PCR conditions are provided on Table 4.3. It has to be noted that in my preliminary screen, the primer pair of LCO_1490/HCO_2198 [242] and C1J_1718/C1N_2191 [243] that were used to amplify *COI* regions of odonates and other invertebrates in Chapter 2 and 3 failed to amplify the bed bug's *COI*.

Samples that passed the quality check were then screened for *Rickettsia* infections using *Rickettsia* specific primers based on the *16S rRNA* gene: Ri170_F/Ri1500_R and the citrate synthase gene (*gltA)*; RiGltA405_F/ RiGltA1193_R (Table 4.3). The conditions were same as described in Table 4.3, save the annealing temperature was changed to 54^{o}C, the conditions were adapted from Pilgrim *et al.* [175]. The DNA sequences of both genes were deposited in the European Nucleotide Archive (ENA) at EMBL-EBI database.

4.3.4 Estimating the phylogenetic affiliation of *Rickettsia*

The *16S rRNA* and *gltA* amplicons from PCR assays were cleaned with the ExoSAP-IT kit (E1050, New England Biolabs, US) and Sanger sequenced. The sequence chromatograms were trimmed and edited in UGENE [258]. All the sequences were exported to fasta format and searched against other *Rickettsia* strains on NCBI database to find close relatives ascertained by BLAST homology. The sequence of these markers from closely related strains from other invertebrate hosts were retrieved and estimated for the relatedness. Other *Rickettsia* strains from other clades, e.g., *Rickettsia bellii* and vertebrate pathogens were selected to represent the sister group to the torix clade. *Occidentia massiliensis* was use as the outgroup for both topologies. All the selected sequences were aligned with *Rickettsia* sequences in this study using MUSCLE algorithm with its default setting in MEGA X [245, 246]. The Maximum Likelihood phylogeny for both genes were estimated in MEGA X with

1000 rapid bootstrap replicates under T92+I and K2+I model for *gltA* and 16S gene respectively.

4.3.5 Transmission mode

To investigate the vertical transmission mode of torix *Rickettsia*, I used two bed bug lab populations, S1 and F4, in which I found the sporadic infections with infected and uninfected individuals throughout the populations. Males and females were randomly selected to establish 65 and 49 mating pairs for S1 and F4 respectively, from which the offspring were reared. The parents and 5-10 randomly selected first instar nymphs were screened for torix infection status using the PCR assays as described above. The first-instar nymphs were tested individually to gain insight into vertical transmission efficiency, and whole bodies were used for template. Then, the impact of parental infection status (mother infected, father infected) on progeny infection status was assessed.

4.3.6 Establishment of iso-female lines and bed bug culture

From the transmission mode experiment above, the infection status of offspring from each mating pair was revealed. Four *Rickettsia*-free and four *Rickettsia*-infected isolines from each of the two populations were used for crossing to establish isofemale lines which they then would be kept under constant conditions. New generations were set up regularly, i.e., at a 6- to 8-week interval. Each new generation was started with randomly picked virgin female and virgin male. All bed bugs were maintained in a CT room at 26±1°C, at about 70% relative humidity with a cycle of 12L:12D. All individuals in this study were virgin prior to experiments. The feeding, maintenance and generation-of-virgin-individual's protocols follow Reinhardt *et al*. [327]

4.3.7 Endosymbiont tropism

To localise torix *Rickettsia* and other symbionts within the *C. lectularius* body, I used the FISH technique adapted from Sakurai *et al*. [12] and Pilgrim *et al*. [175]. The bacteriome and reproductive tissue in virgin male and female adults were investigated, as well as the whole body of first instar nymphs from the torix-free and

torix-infected F4 and S1 lines. Tissues were dissected in 0.5M PBS at pH 7.4 and preserved immediately in Carnoy's solution (chloroform: ethanol: glacial acetic acid = 6:3:1) overnight. The nymphs were preserved in the solution without dissection. All tissue samples were cleared by incubating in 6% H_2O_2 in ethanol for 12 hr, for the whole-body nymph was incubated until the body was transparent (up to 24-48 hr). I then used a tungsten micro-needle to make micropores in the nymph cuticle to allow the fluorescent probes to pass through the cuticle during the hybridization step. The samples were hybridised by incubating the tissues overnight in a hybridization buffer (20mM Tris-HCl pH 8.0, 0.9M NaCl, 0.01% Sodium dodecyl sulfate 30% formamide) with 10 pmol/ml of these symbionts rRNA specific probes for *Rickettsia* [10], *Wolbachia* [6] and Gamma proteobacteria (BEV-like symbiont) [6] (Table 4.3). Nuclei fluorescent staining, Hoechst 33342 (H1399, Invitrogen, Carlsbad, USA), was used to visualise the bed bug tissues. After incubation, tissues were washed in buffer (0.3M NaCl, 0.03 M sodium citrate, 0.01% sodium dodecyl sulfate) and mounted onto a slide using VECTASHIELD® Antifade (H-1000, Vectorlabs, UK) as a mounting medium. Slides were then observed under a confocal microscope, 880 Bio AFM (on 880 LSM platform, ZEISS, Germany).

Table 4.3 PCR primer and fluorescent probe sequences that were used in Chapter 4 and 5. All the fluorescence probes are labelled with a fluorophore, in the square brackets, at 5' end except *RickB1* probe, labelled at 3' end. All the primers were used in the following PCR conditions; initial denaturation at 95 °C for 5 min, followed by 35 cycles of denaturation (94°C for 30s), annealing (Tm°C for 30s), extension (72°C for 50s), and a final extension at 72°C for 7 min. The annealing temperature was varied by the primers.

Target organisms, gene	Primer/ probe Name	Sequence (5'-3')	Tm (°C)	Product size (bp)	Ref.
Bed bug and cimicid allies: PCR primers					
Cytochrome c oxidase subunit I, ***COI***	*C1J_1718*	GGA GGA TTT GGA AAT TGA TTA GT	52	480	[242]
	HCO_2198	TAA ACT TCA GGG TGA CCA AAA AAT CA			[243]
***Rickettsia*: PCR primers**					
16S ribosomal RNA, ***16S***	*Ri170_F*	GGG CTT GCT CTA AAT TAG TTA GT	54	1.1k	[175]
	Ri1500_R	ACG TTA GCT CAC CAC CTT CAG G			
Citrate synthase, ***gltA***	*RiGltA405_F*	GAT CAT CCT ATG GCA	54	786	[175]
	RiGltA1193_R	TCT TTC CAT TGC CCC			
BEV-like symbiont: PCR primers					
16S ribosomal RNA, ***16S***	*BEVF*	GCA CAA GGG AGG TTG CTC CCC	57	420	[6]
	BEVR	CAG CAA GGT TAT TAA CCT TAC TG			
DNA gyrase subunit B, ***gyrB***	*CLBEV1F*	CAC GGG GTA GAT ACC GAT TA	54	310	*
	CLBEV1R	ATG GCG TCT TTA ACT GTC AC			
Endosymbionts: rRNA probes					
γ-proteobacteria	*CimexSec1229R*	[AlexaFluor555]-TTG CTC TCG CGA GGT CGC TT			[6]
Rickettsia	*RickB1*	CCA TCA TCC CCT ACT ACA-[ATTO 633]			[10]
Wolbachia	*TsWo1187Rl*	[AlexaFluor488]-CTC GCG ACT TTG CAG CCC A			[6]
	TsWol944R	[AlexaFluor488]-AAC CGA CCC TAT CCC TTC G			

* The primers are designed in this this study

4.4 RESULTS

4.4.1 Incidence of torix *Rickettsia* in *C. lectularius* lab populations

DNA extractions from each *C. lectularius* population and all cimicid allies passed QC, with good amplicons in the COI amplification. Thirteen out of twenty-one *C. lectularius* populations tested positive with *Rickettsia* infection with both *16S rRNA* and *gltA* primers. These populations have their origin in Africa, mainland Europe and UK (Table 4.4). Both male and female individuals were found to be infected in most cases where infection was detected. In the initial screen, only the female individual was scored as infected in populations S1 and BG1; however, infection in males was observed in both populations on deeper screening.

4.4.2 *Rickettsia* in other cimicid species

DNA samples of bed bug allies passed the quality check procedure, and these were tested for *Rickettsia* infection in PCR assays. The PCR results indicated that just only one species, *Afrocimex constrictus* (subfamily Afrocimicinae), was positive with *Rickettsia* infection in all three samples (Table 4.5). However, the rickettsial PCR only produced *gltA* amplicons for this species, despite repeated attempts at *16S* amplification.

Table 4.4 *Rickettsia* infection in *C. lectularius* stock populations in Bayreuth lab. Males in the brackets (M) indicate the infections were detected later when these populations were screened with more individuals. Asterisks in population name indicate that these populations were selected for transmission mode experiment and established as the iso-female lines.

Population name	Origin of place	Year established in Sheffield	Male /Female (M/F) positive
H1	Budapest, Hungary	2010	neither
C1	Coventry, UK	2010	neither
Bayer	Germany	2006	neither
S1*	London School of Tropical Medicine	1996	(M)/F
London heavy	London, UK	2006	neither
F11	London, UK	2006	neither
F4*	London, UK	2006	M/F
F10	London, UK	2006	neither
YMCA	London, UK	2010	neither
K12	Nairobi, Kenya	2008	M/F
K4	Nairobi, Kenya	2008	M/F
K22	Nairobi, Kenya	2010	M/F
K25	Nairobi, Kenya	2010	M/F
K26	Nairobi, Kenya	2010	M/F
K19	Nairobi, Kenya	2010	M/F
K20	Nairobi, Kenya	2010	M/F
K7	Nairobi, Kenya	2008	M/F
K5	Nairobi, Kenya	2008	M/F
K30	Nairobi, Kenya	2010	M/F
BG1	Sofia, Bulgaria	2010	(M)/F
K17	Watamu, Kenya	2010	neither

Table 4.5 *Rickettsia* infection in cimicid allies. Only *Afrocimex contrictus* was positive for the *Rickettsia* in the PCR screen.

Subfamily	Species	*N*	Locality	Year of collection	Infection
Afrocimicinae	***Afrocimex constrictus***	**3**	**Kenya**	**2005**	**+**
Primicinae	*Bucimex chilensis*	1	Chile	2013	-
	Primicimex cavernis	1	Mexico	2015	-
Haematosiphoninae	*Ornithocoris pallidus*	1	USA, South Carolina	2010	-
	Acanthocrios furnarii	1	Brazil	2010	-
	Psitticimex uritui	1	Argentina	2008	-
	Cyanolicimex patagonicus	1	Argentina	2003	-
	Cimexopis nyctalis	1	USA	2016	-
	Hesperocimex sonorensis	1	Mexico	2017	-
	H. coloradensis	1	Los Alamos County, N.Mex.	1971	-
Cimicinae	*Paracimex inflatus*	1	Kavieng, Papua New Guinea	1966	-
	P. borneensis	1	Borneo	2015	-
	Cimex pipistrelli	1	Hanau, Germany	2004	-
	C. hemipterus	1	Taiwan	<1966	-
	C. hirundinis	1	Switzerland	N/A	-
Cacodminae	*Aphrania elongata*	1	Senegal	2012	-
	A. vishnou	1	Phnom Penh, Cambodia	1952	-
	Cacodmus villosus	1	Kenya	2005	-
	Loxapsis malayensis	1	Tasik Bera, Pahang, Malaysia	1962	-
	Leptocimex duplicatus	1	Israel	2002	-
	L. boueti	1	N/A	N/A	-

4.4.3 Estimating the phylogenetic affiliation of *Rickettsia*

The *16S rRNA* sequences alignment of all *Rickettsia* strains from the *C. lectularius* populations indicated that these strains were identical (based on 985 bp sequence length information, accession number; LR828195). The topology of *16S rRNA* gene showed the *Rickettsia* strain of *C. lectularius* is affiliated in torix group (Figure 4.3), which is closely related to *Rickettsia* endosymbiont of *Nephotettix cinticeps* leafhopper. These strains are monophyletic (albeit with low bootsrap support) with other *Rickettsia* endosymbionts of glossiphoniid leeches, i.e., *Hemiclepsis marginata*, *Torix tukubana* and *T. tagoi*, and also potentially form a sister clade with a *Rickettsia* endosymbiont of a common blue damselfly (*Enallagma cyathigerum*) and a *Rickettsia* endosymbiont of *Culicoides newsteadii* biting midge.

The alignment of those *gltA* sequences (746 bp) of *Cimex lectularius* also showed no variable sites across all *Rickettsia* positive populations. Moreover, the sequences of the *gltA* amplicons from both *C. lectularius* and *A. constrictus* (accession numbers; LR828196-LR828197) were also identical. On *gltA* topology, these strains are closely related to a *Rickettsia* endosymbiont of the flea, *Nosopsyllus laeviceps* and sister of the clade containing *Rickettsia* endosymbiont of *Culicoides newsteadii* biting midge (Figure 4.4).

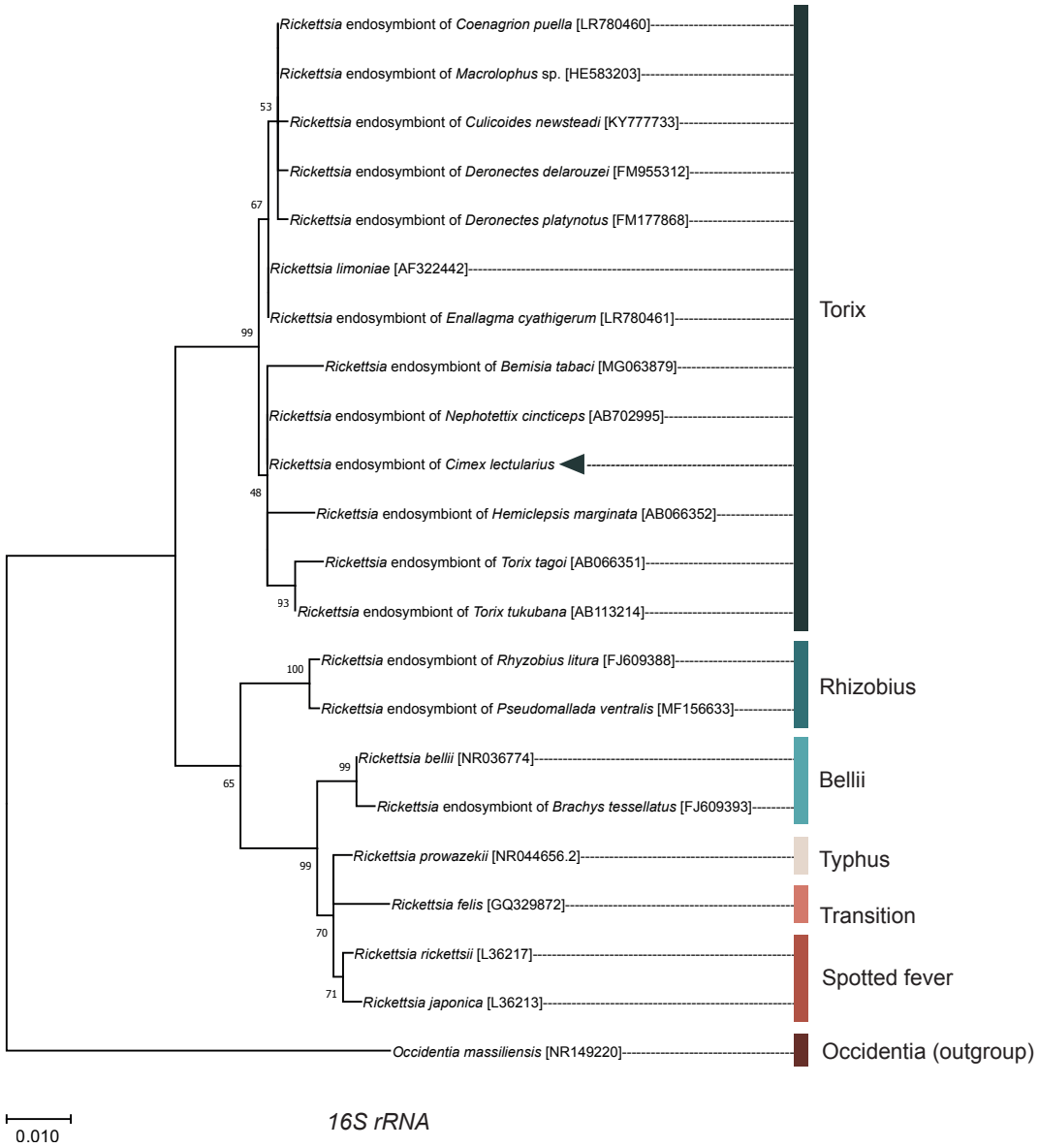

Figure 4.3 Maximum likelihood phylogenetic trees generated from *16S rRNA* gene (sequences showing the position and relatedness of *Rickettsia* endosymbiont of *C. lectularius* in this study (arrowhead) with other relative strains that obtained from NCBI (the GenBank accession numbers are in the square brackets). The bootstrap values expressed as the percentage of 1000 replicates are shown at the nodes. The bar indicates substitution of nucleotides per position.

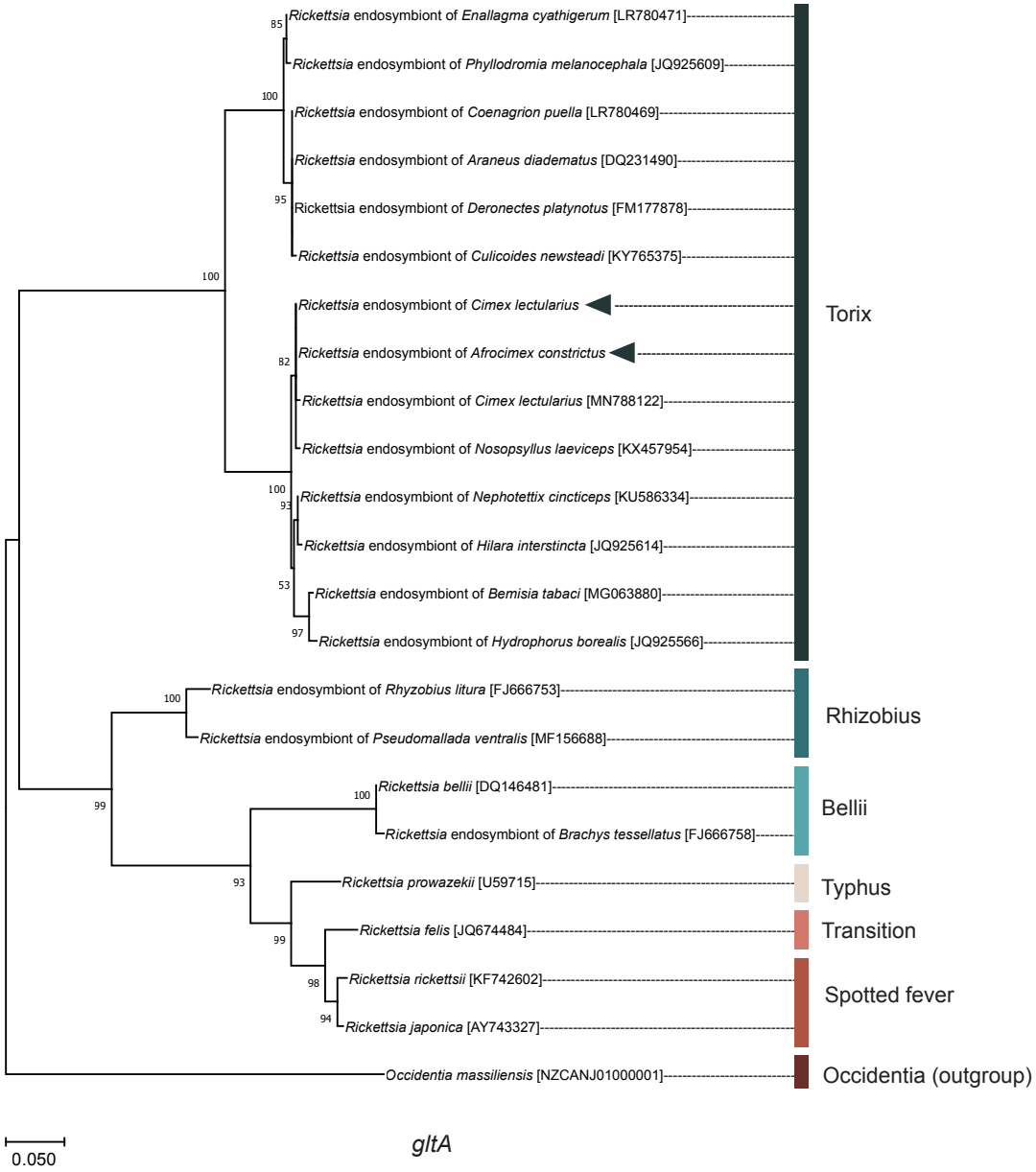

Figure 4.4 Maximum likelihood phylogenetic trees generated from *gltA* gene sequences showing the position and relatedness of a *Rickettsia* endosymbiont of *C. lectularius* and *A. constictus* found in this study (arrow head) with a *Rickettsia* endosymbiont of *C. lectularius* from Potts *et al*. [325] (accession no. MN7881222) and other relative strains that obtained from NCBI (the GenBank accession numbers are in the square brackets). The bootstrap values expressed as the percentage of 1000 replicates are shown at the nodes. The bar indicates substitution of nucleotides per position.

4.4.4 Transmission mode

All 49 crosses of the F4 line produced offspring, while 55 out of 65 crosses from line S1 were successful. The parents who produced the offspring were test for *Rickettsia* infection. The frequency of infected individuals in the F4 population was lower than that of S1 (F4: 14 of 49 mother, and 20 of 49 fathers carried the symbiont; S1 population, 48 of 55 mothers, and 50 of 55 fathers carried the symbiont). The *Rickettsia* infection status for each family was categorised into four groups according to parental infection status.

Rickettsia transmission to progeny was consistently observed where either both parents (R+ x R+) or just the mother (R+ x R-) tested positive for torix *Rickettsia* (R+ x R+: F4: 8 crosses, S1: 45 crosses; R+ x R-: F4: 6 crosses, S1: 3 crosses, Figure 4.5). In these cases, all 310 tested nymphs had acquired infection, indicating vertical transmission through females was highly efficient (Binomial confidence intervals for vertical transmission efficiency 0.988-1.000). No progeny tested positive for *Rickettsia* infection in families where only the father was infected (R-x R+: F4: 12 crosses, S1: 5 crosses), nor were any *Rickettsia* positive individuals recovered from crosses where neither parent was infected (R- x R-: F4: 23 crosses, S1: 2 crosses). These data indicate that maternal infection is necessary and sufficient for the presence of *Rickettsia* in progeny, and there is no evidence of paternal inheritance.

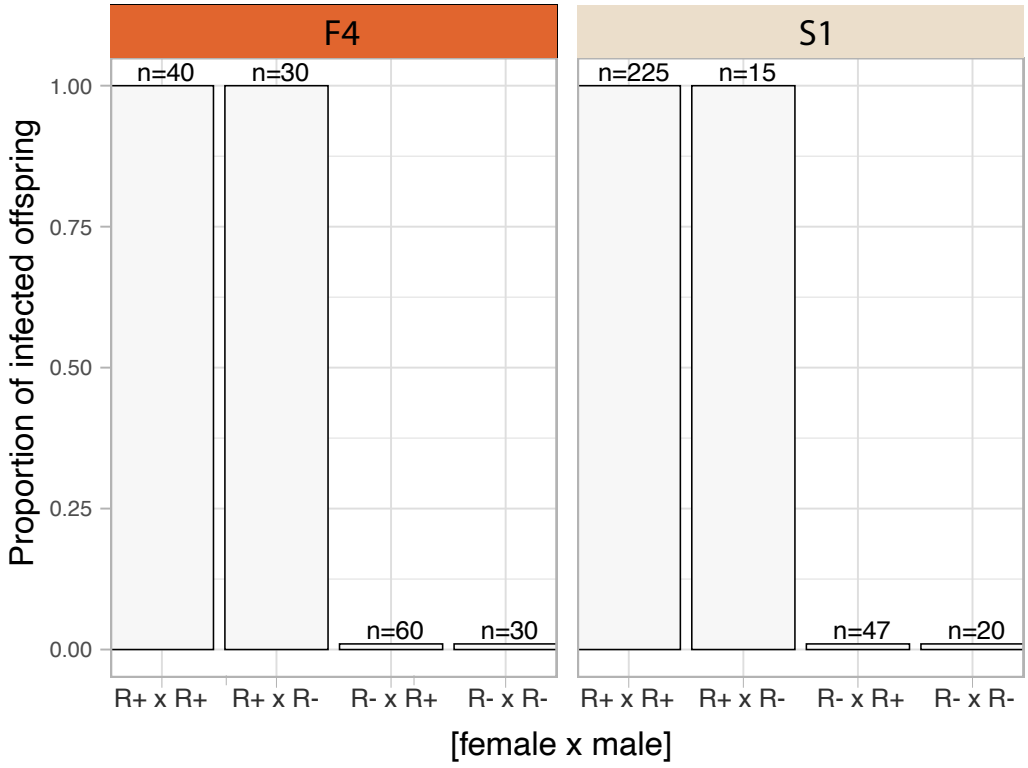

Figure 4.5 Transmission mode of *C. lectularius* associated *Rickettsia*. The bars indicate proportion of infected offspring in each crossing group. The four crosses were sorted by infection status of the parents, female x male (R+ x R+: 8 crosses for F4, 45 for S1; R+ x R-: 6 crosses for F4, 3 for S1; R-x R+: 12 crosses for F4, 5 for S1; for R- x R-: 23 crosses for F4, 2 for S1). Number of tested offspring are given on the bars. None of infected offspring was observed from *Rickettsia*-free mother groups (R- x R+ and R- x R-).

4.4.5 Localization of torix *Rickettsia* in adults

FISH detected *Rickettsia* throughout ovaries and bacteriome tissues (Figure 4.6, 4.8 and 4.9 D - E). In adult females, the distribution of the *Rickettsia* signal was intense in the trophic core of the tropharium and in oocytes. Alongside *Rickettsia* in this area, the filamentous BEV-like symbiont and *Wolbachia* were also observed. In comparison to the other symbionts, *Rickettsia* were more widely distributed in the ovaries. I observed them also in nurse cells and follicular epithelial cells (Figure 4.6 C-D). *Rickettsia* signals were absent in *Rickettsia*-free bed bugs (Figure 4.7). Symbiont signals failed to be detected in male reproductive tissues in this study, except the somatic bacteriome where only the BEV-like symbiont and *Wolbachia* are observed (Figure 4.8).

4.4.6 Localization of torix *Rickettsia* in first instars

Rickettsia signals were found in somatic tissue of the abdominal areas of first instar bed bugs, alongside the BEV-like symbiont (Figure 4.9). At low magnification, the strongest signal (green colour) was emitted by *Wolbachia* reflecting the intense infection of bacteriome organs (Figure 4.9 B). *Rickettsia* and the BEV-like symbiont, however, could also be seen at this low magnification but poorly resolved. At higher magnification, *Rickettsia* is clearly visible in the bacteriome alongside *Wolbachia* and the BEV-like symbiont as all the three signals were reliably present in this tissue (Figure 4.9 D - E). In *Rickettsia*-free samples only the signals of *Wolbachia* and the BEV-like symbiont were present, while *Rickettsia* signals were absent (Figure 4.9 C).

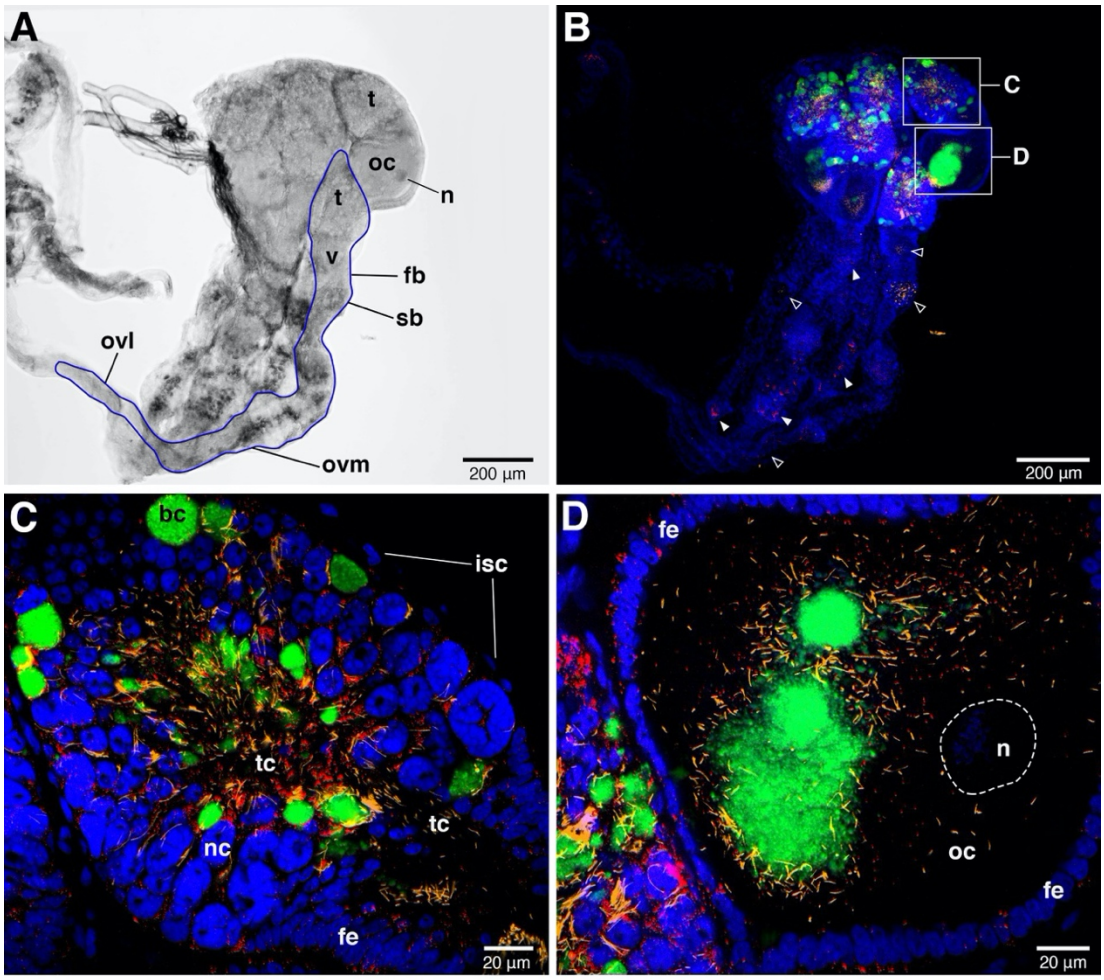

Figure 4.6 FISH images of adult *Rickettsia*-infected female ovaries. **A:** Bright field image of one-sided ovaries. The blue line represents the outline of one ovariole. n = nucleus of oocyte, t = tropharium part, v = vitellarium part, fb = follicular body, oc = oocyte, sb = syncytial body, ovl = lateral oviduct, ovm = mesodermal oviduct. **B**: FISH shows the presence of the three symbionts, i.e., *Wolbachia* (green), BEV-like symbionts (yellow) and *Rickettsi*a (red) in ovaries. Blue colour represents nuclei of bed bug cells. The signals of three symbionts are concentrated in tropharium areas. Small rectangles indicate the magnified fields of tropharium and vitellarium portions, showing in **C** and **D**, respectively. *Rickettsia* (filled triangles) and BEV-like symbionts (open triangles) are also detected in syncytial body and mesodermal oviduct at low densities. **C**: Enlarged detail of the tropharium portion. It is covered by a membrane of inner sheath cells (isc). The three symbionts distribution can be detected at very high density all along the trophic core (tc) area. *Wolbachia* are likely packed in bacteriocytes (bc) which are distinctive to the adjacent nurse cells (nc), while *Rickettsia* and filamentous BEV-like symbionts are more scattered. **D**: Enlarged detail of vitellarium portion. All three symbionts invade the oocyte, forming a cluster at the posterior pole of the oocyte. *Rickettsia* signals are scattered insertions in the follicular epithelium (fe) of the oocyte.

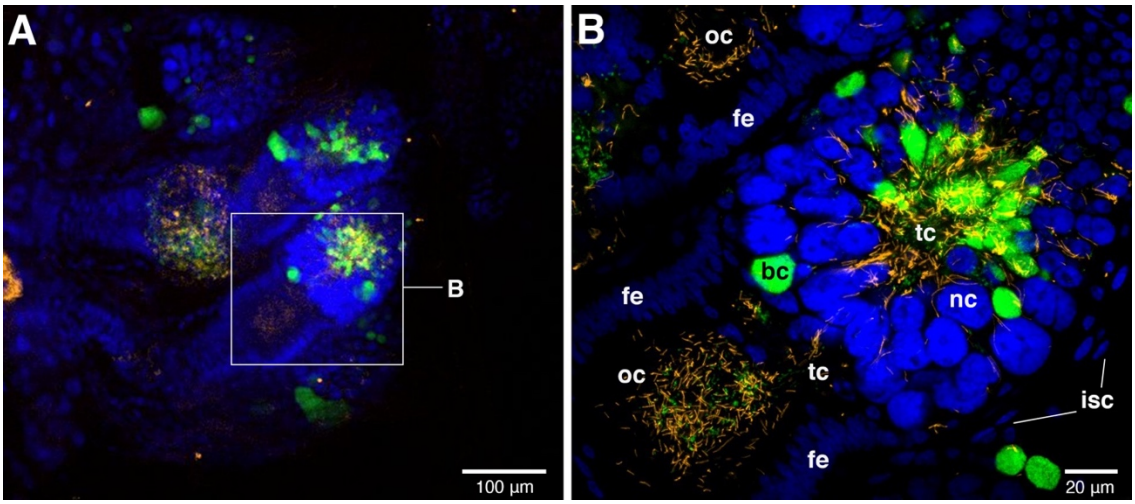

Figure 4.7 FISH images of ovaries from *Rickettsia*-free female adult **A**: Ovaries of torix-free bed bug. Only *Wolbachia* (green) and BEV-like symbionts (yellow) are present. Blue colour represents nuclei of bed bug cells. The small rectangle represents the tropharium and vitellarium parts of one ovariole showing in **B**. **B**: Enlarged field of partial upper ovariole. It is covered by a membrane of inner sheath cells (isc). *Wolbachia* are packed in bacteriocyte (bc) before entering the oocyte (oc), while BEV-like symbionts are loosely diffused. The signals of the two symbionts are intensive in trophic core (tc) and in oocyte. None of *Rickettsia* signals (red) are detected here. fe = follicular epithelium, nc = nurse cells.

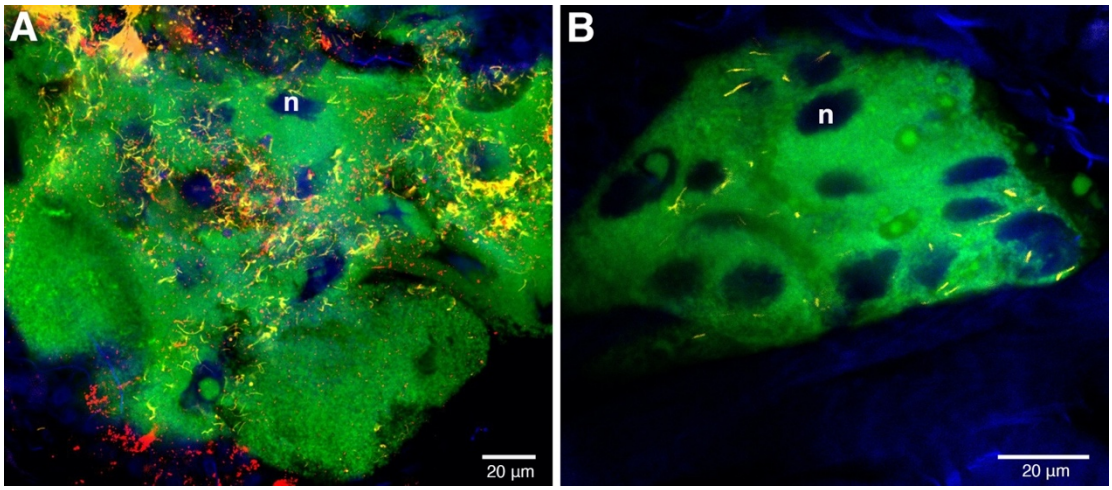

Figure 4.8 Bacteriome of adult *C. lectularius.* **A**: FISH revealed co-occurring of *Wolbachia* (green), filamentous BEV-like symbiont (yellow) and *Rickettsia* (red) in *Rickettsia*-infected female. The boundary of bacteriome is easily defined by the present of *Wolbachia* signals in multiple bacteriocytes with multiple oval-shaped nuclei. **B**: Bacteriome of *Rickettsia*-free male. The signals of *Rickettsia* are absent, with only *Wolbachia* and BEV-like symbionts observed. n = nucleus of bacteriocytes.

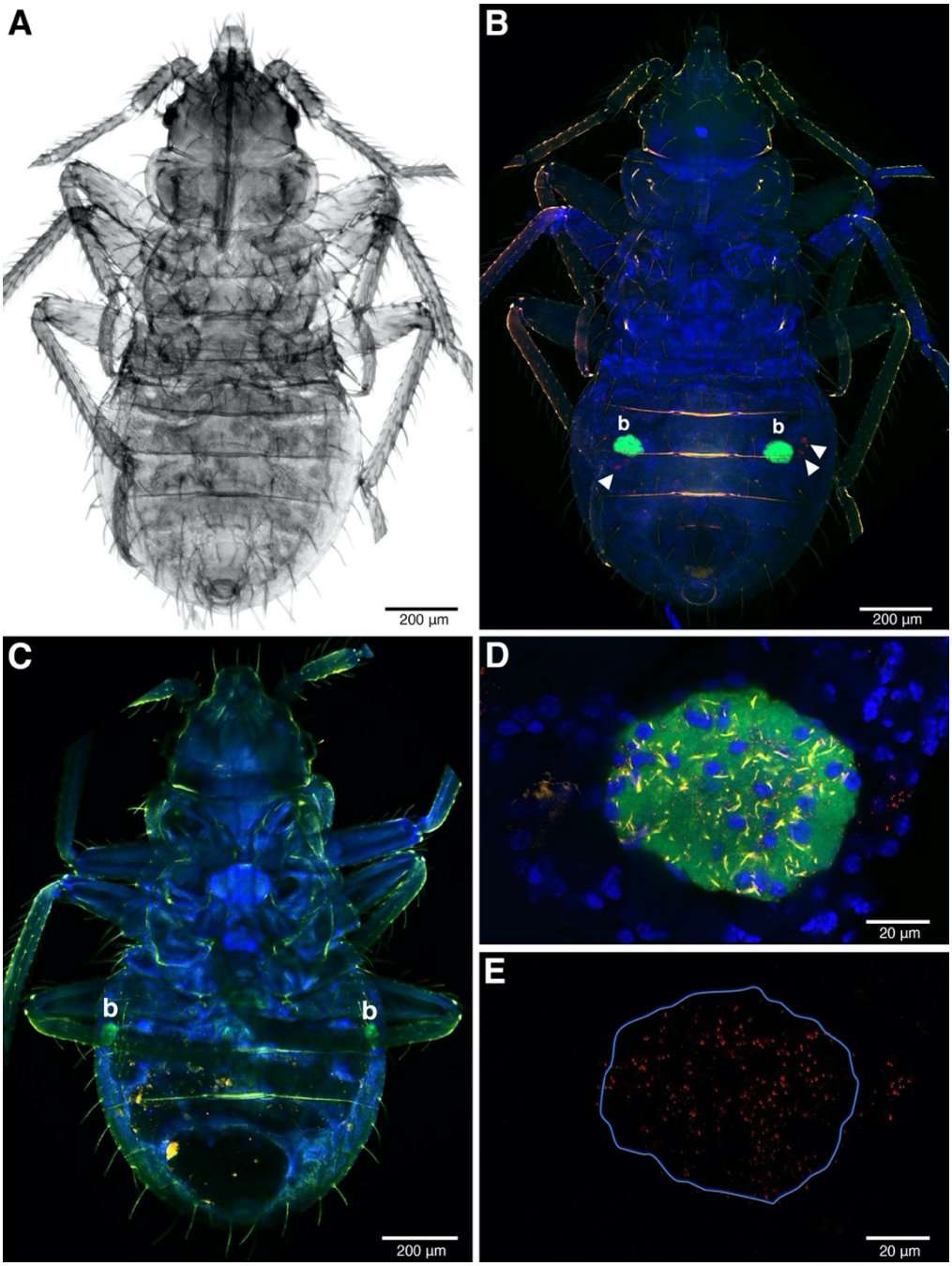

Figure 4.9 FISH image of the whole-mounted first instars. **A**: The nymph under transmitted light. **B-C**: FISH detection of symbionts in *Rickettsia*-infected (**B**) and *Rickettsia*-free instars (**C**). The ball-shaped in green colour represents strong *Wolbachia* signals indicate where bacteriome allocation in abdomen. *Rickettsia* infection showing in red where the filled arrowhead present in **B** but absent in **C**. *b* = bacteriome. **D**: Higher magnification of bacteriome of *Rickettsia*-infected instar. All the three symbionts can be detected in this organ. *Wolbachia*, BEV-like symbionts and *Rickettsia* are in green, yellow and red respectively. The blue colour represents nuclei of bed bug cells. **E**: The same field as **D** but only the *Rickettsia* channel remains. The blue line indicates the bacteriome boundary.

4.5 DISCUSSION

4.5.1 Torix *Rickettsia* in *Cimex lectularius* and another cimicid

This study revealed the frequency and transmission biology of torix *Rickettsia* in a blood-feeding insect. *Cimex lectularius* are notorious pests of humans [287]. In pest management field, it is important to understand bed bug biology in all the possible aspects in order to cope with its infestation [287, 328]. Studying endosymbiont association is a potentially powerful tool to overcome its infestation [329]. Generally, the majority of studies investigate its primary endosymbiont '*Wolbachia'*, because this symbiont is known to have the strong vital effects on the bugs [284, 310, 315]. In this study, I have assessed another facultative endosymbiont '*Rickettsia*' to extend our perspectives on endosymbiont biology and symbiont-host interactions in this important pest species.

The detection of a single torix *Rickettsia* strain in cosmopolitan bed bugs indicates that one main strain of this symbiont circulates in this species worldwide. This finding also appears in the recent study of Potts *et al.* [325]. The *Rickettsia* strains was investigated in *C. lectularius* from UK and USA field collections, all of which reveal identical *gltA* haplotype (accession no: MN788122 as the representative strain sequence) which is 98.39% similar (NCBI BLAST) to the strain found in my study. There are 6 SNP detected (3 at the beginning of 5' and another 3 in the 3' end) between the sequences from the two studies. These positions correspond with the priming site for PCR, indicating the differences may be artefactual, and simply reflect the primer sequence used for amplification. Thus, it is likely that the *Rickettsia* strain found in Potts *et al*. [325] is actually identical to the torix *Rickettsia* strain in this study.

Whilst a single strain of *Rickettsia* was present, not all individuals carried the *Rickettsia*. It was observed that some of the laboratory cultures lacked *Rickettsia*. Interestingly, lines F4 that was isolated in 2006 in Sheffield all carried *Rickettsia*, but these same lineages were polymorphic for infection when samples were obtained in 2018. Thus, the infection had segregated during laboratory passage. These data contrast with the worldwide maintenance of the essential *Wolbachia* symbiont of *C. lectularius* (wCle) [6, 312].

What is the origin of *Rickettsia* infection in bed bugs? The horizontal transmission of symbionts in a host shift event is a classical theory to explain the distribution of single endosymbiont strain that then spreads across insect host populations. The secondary endosymbiont could occasionally undergo horizontal transmission by unknown route [330]. In such cases, *C. lectularius* are the ectoparasites of warm-blood animals, so this could be by *Rickettsia*-free bed bugs acquiring the symbionts by feeding on *Rickettsia*-infected hosts. Alternately, infection could be derived from uncharacterised parasitoid insects. However, the initial source and route of acquisition are unclear. In terms of spread, one possible scenario could be that the *Rickettsia* have symbiosed the host since the *Cimex* ancestors before the speciation and colonisation has begun. Alternately, it may have arisen more recently, and then spread associated with intercontinental movement of bed bugs associated with human movement.

It was also noted that torix *Rickettsia*, identical at the genetic loci sequenced, was also found in the bat bug (*Afrocimex constrictus*). This phenomenon is similar to the sharing of torix strains by two damselflies from different genus, *Coenagrion puella* and *Erythromma najas* (Chapter 2). These data indicate that the endosymbiont does jump across host species, but whether this is direct, or through the wider community, is not established.

4.5.2 Maternal inheritance

It is typical for inherited endosymbionts to be transmitted maternally between host generations [331, 332], and bed bug associated torix *Rickettsia* was maternally inherited in the assays presented. In the experiments presented, maternal inheritance occurred with 100% fidelity – all 310 progenies from infected females carried the infection. However, the segregation of the symbiont during 12 years of laboratory passage indicates some level of inefficient maternal inheritance. Thus, I can conclude that whilst vertical transmission through females is very high, it does not occur with 100% fidelity.

In contrast, no paternal transmission was observed in this study, despite paternal males carrying infection (from the PCR assays), and previous evidence of

Rickettsia in male sperm containers [324]. However, the symbiont could not be passed to their progeny, suggesting that bed bug sperm morphology might not yet be suitable for the *Rickettsia* carriage at this time point of their co-evolutionary process. The situation contrasts with the leafhopper- associated *Rickettsia* case, In *Nephotettix cincticeps,* torix *Rickettsia* can transmit biparentally, with 70% father-progeny and 100% mother-progeny transmission rates. The *Rickettsia* are found in nuclei areas of sperm counterparts without interrupting the sperm functions [249]. Notably, the *Rickettsia* in the leafhopper has the capacity for intranuclear infection, which likely is necessary for paternal inheritance. Beside the plant hopper, paternal transmission is also observed in a tsetse fly-associated *Sodalis* symbiont [333], a group of gamma proteobacteria. Paternal inheritance has also been noted for symbionts in the genus *Megaira*, the sister taxa to *Rickettsia* [334]. Generally, intranuclear infection is present in a range of endosymbiotic bacteria, e.g., *Nucleococcus*, *Chlamydia*, gamma and alpha proteobacteria [335]. This trait is observed quite widely in the Rickettsiacae, but is labile, being present in some symbioses but not others [335].

Paternal inheritance of endosymbionts is also known to be limited by the host's sperm counterpart capacity [336]. On the other hand, the finding of a strong maternally inherited pathway in this study, bed bug and *Rickettsia* potentiates conflict with respect to host sex, as males are evolutionary dead ends for maternal inheritance [337, 338]. This will be further invested in the next chapter.

4.5.3 Symbionts in the bacteriomes

Insects commonly live mutualistically with endosymbionts to facilitate each other. Insect hosts provide a bacteriome as an organ for harbouring their endosymbionts in many cases [6, 11, 316, 332, 339]. In previous histological studies, the bacteriocytes of *C. lectularius* are harbourage of two bacteriome-associated endosymbionts, the primary endosymbiont *Wolbachia* and secondary BEV-like endosymbiont [6].

In this study, I additionally examined the tropism of *Rickettsia*, alongside *Wolbachia* and BEV-like symbionts, in these organs. Overall, the bacteriome FISH

image indicates a high signal intensity for *Wolbachia*. *Rickettsia* was present in the bacteriome, but the three symbionts were spatially intermixed within the bacteriomes from the z-stack layer images. This result contrasts with the localization the symbiont community in the leafhopper, *N. cincticeps* [11]. There are three endosymbionts involved in this community. Two are major essential bacteriome-associated endosymbionts, *Sulcia* bacterium and β-proteobacterium. They live separately in two different morphological bacteriocytes that located in the outer and inner region of the bacteriome, respectively. Although, this study lacks a visual evidence of *Rickettsia* infection in other somatic tissues (e.g., haemolymph, excretory, digestive and immune systems), the consistent detection of *Rickettsia* infection in head and leg materials during the transmission experiment indicate the infection is diffusely present. *Rickettsia* infection in legs is likely to be derived from haemolymph, as has been reported in other *Rickettsia*-host systems [340-342] including in *N. cincticeps* [196].

The specific territory of the symbiont infection in the bacteriome might imply a more specific function of those symbionts for their host [343, 344]. Investigating in-depth in the bed bug bacteriomes might help to understand the distribution of these three endosymbionts and could potentially predict the biological impacts of these endosymbionts on the host or the interaction effects among themselves.

The presence of *Rickettsia* in bacteriocytes presents a route for achieving maternal inheritance. *Wolbachia* vertical transmission in this system is associated with movement of a bacteriocyte towards the ovary, and then fusing to deliver a symbiont cargo (Figure 4.6 C-D, 4.7 B and Appendix Figure S1 B and D with higher magnification and a different layer of bacteriocytes from z-stack image). The presence of *Rickettsia* in the bacteriome likely allows this symbiont to hitch-hike to the ovary to gain vertical transmission. However, having established in the ovary, *Rickettsia* and *Wolbachia* show distinct patterning, with *Wolbachia* retained in discrete clumps, but *Rickettsia* being more diffuse.

4.5.4 Interaction and the potential role of the three symbionts

The co-existence of these three endosymbionts within the same host might have the direct or indirect effects towards the host biology, or alternatively an interaction effect within among themselves [345]. This has not yet been studied. However, the presence of the different organisms within the same habitat provides an arena for positive and interference outcomes [346].

Wolbachia is well recognized as B-vitamin supplement provider for *C. lectularius* whereas the other two secondary endosymbionts, BEV-like symbiont and *Rickettsia* have not yet been documented [6]. BEV-like symbionts are thought to be a non-essential endosymbiont of *C. lectularius* as noted by Hosakawa *et al.* in 2010 [6] where it is sporadically found in *C. lectularius* populations [6, 311, 347]. The BEV-like baterium appears to be a motile symbiont of *C. lectularius* in description of Louise *et al.* in 1973 [348]. Moreover, Arkwright *et al*. in 1921 [322] have also described the thread-like motile phase of *Rickettsia* found in the bug under light microscopic study and suspected it as parasitic endosymbiont, and likely represents BEV-like symbiont associated with *C. lectularius.* Thus, it could be said under this uncertain scenario that, the potential parasitic endosymbionts mentioned in Arkwright *et al*. in 1921 [322] refer to BEV-like bacterium or *Rickettsia* or it could be both of the symbionts that have been showing a mildly parasitic interactions to the insect host.

4.5.5 Could *Rickettsia* alter bed bug biology?

The biological impact of *Rickettsia* on bed bug biology is currently not known. Few recent examples of the biological impact of torix *Rickettsia* can be found in glossiphoniid leeches and *Cerobasis* booklouse cases. In the former case, they demonstrated that torix *Rickettsia* had a direct effect on the body size of the three leech host species, with infected individuals being larger [135]. The second case, torix *Rickettsia* seemed to be associated with a parthenogenesis induction [10]. Recent study has examined the genome sequence of the endosymbiont of *Culicoides newsteadi,* the relative strain to bed bug-associated *Rickettsia.* No evidence of the capacity for positive facilitation, e.g., B-vitamin gene provisioning capacity, was

observed. However, the genome possesses unique features of genes that potentially associated with host invasion and adaptation [175].

Commonly, investigating the impact of a symbiont requires comparison of infected and uninfected lineages, identical in genetic background. These are usually obtained through antibiotic treatment, but this is not easily achieved in host species where there is an obligate symbiont association. The polymorphism observed in F4 and S1 in this chapter does allow such a comparison, as natural segregation in lab passage has produced isolines with and without *Rickettsia*. These isolines will then be used for determining the effects of torix *Rickettsia* carriage in the next chapter.

4.6 CONCLUSION

In this Chapter I revealed more in-depth perspective of bed bug associated *Rickettsia* biology. The study observed the prevalence of *Rickettsia* in *Cimex lectularius* populations, with 61.9% of screened lab populations carrying this symbiont. The strains found in the cosmopolitan populations are likely identical based on the *16S rRNA* and *gltA* genes, and affiliated in 'torix' clade. This strain is also found in *C. lectularius* allied species, the African bat bug, *A. contrictus.* The reason why these two species shared the potential similar *Rickettsia* strain is unclear. This bed bug-associated *Rickettsia* is only transmitted via maternal passage; whilst adult males were observed to be infected, they couldn't transmit the symbionts to their offspring. The torix *Rickettsia* was observed in ovaries and bacteriome, which they coinhabit with other endosymbionts, *Wolbachia* and BEV-like symbiont. The strict maternal inheritance suggested *Rickettsia* might play some role in bed bug reproduction or influence some biological impacts of the bed bug hosts. These impacts will be investigated in the next chapter

CHAPTER 5

The impact of torix *Rickettsia* on the development and reproduction of the common bed bug (*Cimex lectularius*)

Publication and author contributions

All results in this chapter, except body size and longevity experiments (Figure 5.5 and 5.6), are in press at *Frontiers in Microbiology* with a bioRxiv pre-print under Thongprem *et al*. in 2020 [282]. Experiments of development time, sex-ratio, fecundity, CI and longevity were completed with the help of Dr Oliver Otti (University of Bayreuth, Germany). Body size measurement was completed by me with advice of Dr Oliver Otti. The experiments above were held at University of Bayreuth, Germany. All molecular experiments and data analysis were completed by me at University of Liverpool.

5.1 ABSTRACT

In the previous chapter I revealed the existence of the facultative *Rickettsia* symbionts in *Cimex lectularius* populations. This bacterium is transmitted via maternal passage and is present in the bacteriome and reproductive tissues of the adult bedbug. Vertical transmission partially aligns the fitness of symbiont and host, and can select for symbiont contribution to host function. Contrastingly, maternal inheritance may select for reproductive parasitism. I here report the results of experiments comparing the biology of infected/uninfected bed bugs from two isolines, examining evidence for the presence of general biological fitness and reproductive manipulation phenotypes. The results did not support the hypothesis that torix *Rickettsia* were acting as reproductive parasites, but did indicate *Rickettsia* infection has negative effects on bed bug fecundity and development time. These results lead to the question of the 'missing factors' maintaining *Rickettsia* in the population.

5.2 INTRODUCTION

When two organisms are living together in symbiosis, the symbiont can have multiple impacts on the biology of the host [346]. Bacterial endosymbionts may provide essential resources or services that benefit host fitness [6, 10, 133, 139, 157], or may place a metabolic cost on the host [110, 141, 195], or could cause a deleterious impact through reproductive manipulations [24, 57, 86, 108, 349]. These effects are varied depending on different species partnerships and the way that endosymbionts are inherited, i.e., biparental, only paternal or only maternal transmissions.

5.2.1 Being maternal inherited symbionts

Vertical transmission through maternal passage aligns the fitness and transmission of the symbionts to female host survival or reproduction [350]. The dependence on female (rather than male) hosts can lead to the evolution of reproductive parasitism [349]. Reproductive parasitism can be expressed in different ways, e.g., distorting sex-ratio of the host populations by inducing feminization [351], a male-killing phenotype [57] or inducing parthenogenesis [90] (See more details and related examples of the reproductive parasitism in Chapter 1 section 1.1.2). Sex-bias towards female may benefit the bacterial symbiont by increasing the chance that the symbionts are in the healthy female hosts to pass their bacterial descendants into new host generations. In contrast, for the host perspective, these phenotypes may reduce general host fitness. Male-killing involves the death of 50% of progeny; parthenogenesis induction prevents sexual recombination. Commonly, sex ratio distorters select for host traits that prevent their action by suppressor genes [349].

In addition, there is another common symbiont-induced reproductive modification in insects, cytoplasmic incompatibility (CI). This phenomenon prevents the host from successfully forming viable embryos when the infected male mates with non-infected females, or with females carrying a different symbiont strain (See more details in Chapter 1 section 1.1.3). These parasitic symbionts only benefit infected females, as the infected females can mate with either infected or non-infected males and they can produce viable offspring, while the symbionts decrease

the fitness of infected male and non-infected female hosts [352]. Cytoplasmic incompatibility was originally described as a phenotype of *Wolbachia* [101], but has since been observed more widely, having been recorded in *Cardinium* and *Rickettsiella* symbionts [111-113, 353].

Negative effects of these symbionts do not always occur in these symbiosis systems, and in many cases carrying a symbiont improves host fitness. A wide variety of 'services' are provided by symbiont e.g., providing protection against natural enemies [155, 195], xenobiotics resistance [354, 355], anabolic roles, such as provision of vitamins/essential amino acids [2, 5, 6, 356, 357]. These phenotypes may occur alongside, or independently, of reproductive parasitism.

5.2.2 The effects of *Rickettsia* carriage

Studies in the last decades have documented the roles of *Rickettsia* endosymbionts on the biology of a few invertebrate hosts. In 1994, it was first discovered that *Rickettsia* in ladybirds (*Adalia bipunctata*) can exist strictly as vertically transmitted endosymbionts of arthropods [57], without a mammalian transmission phase. This *Rickettsia* symbiont induced a male-killing phenotype in the ladybird, which caused female-bias to the population of the insect. This phenotype is lethal to male offspring by preventing the development of male embryos [52, 61]. Another case of reproductive parasitism could be found in parasitic wasps (*Pnigalio soemius*) in which the *Rickettsia* induce the female wasp to become parthenogenesis [90]. However, *Rickettsia* has never been observed as an agent causing cytoplasmic incompatibility events as reported in *Cardinium, Rickettsiella* and *Wolbachia*

In contrast to this parasitic nature, some *Rickettsia* strains have been observed to have a direct benefit for their host. In aphids, *Rickettsia* infection improves host defence against fungal pathogens [155]. Some *Rickettsia* have an essential role in development of early insect oocytes [10] and may influence the growth by increasing the body size of the host [135]. Genome sequence evidence indicates that the *Rickettsia* in the tick *Ixodes pacificus* has a complete pathway for folate biosynthesis, indicating a potential benefit to symbiont presence in this system [123]. In contrast to these positive benefits, some *Rickettsia* strains can influence negative

performance of the host. For example, pea aphids (*Acyrthosiphon pisum*) associated with *Rickettsia* are likely to have lighter body weight and produce fewer offspring than those without *Rickettsia* [12]. However, the roles of *Rickettsia* symbionts are poorly documented when compared to the catalogue of species in which host-*Rickettsia* symbioses have been observed.

A major impediment to discovering the role of *Rickettsia* in host biology is the presence of suitable model host systems. In order to analyse the impact of a symbiont, the biology of *Rickettsia* infected and uninfected host individuals must be compared. This requires first, a host system that is tractable in the laboratory, and second, the presence of symbiont-infected and uninfected individuals to study [6, 358, 359]. These comparisons would need to be made either on a common 'isoline' genetic background, or in a randomized variable background. The presence of infected/uninfected individuals may occur naturally [135] but may also require curing in the laboratory. Even for model species, this may be difficult to achieve for cases where the host has additional symbioses with required microbial partners.

5.2.3 Aims

In the previous chapter, two laboratory populations of the bed bug, S1 and F4, were observed to be composed of both *Rickettsia* infected and *Rickettsia* uninfected individuals. Individual families were isolated in order to establish sublines of each, with and without *Rickettsia*. In this chapter, I use these sublines to compare the biology of *Rickettsia*-infected and *Rickettsia*-uninfected individuals. Within this, I examine evidence of reproductive parasitism (sex-ratio distortion, cytoplasmic incompatibility) and life history impacts (development rate, fecundity, body size and longevity).

5.3 METHODS

5.3.1 Isofemale lines and bed bug culture

Based on the infection status of offspring from the transmission mode experiment in the previous chapter, four *Rickettsia*-free (R-) and four *Rickettsia*-infected (R+) isolines were established from each of populations, F4 and S1. These isofemale lines of known *Rickettsia* infection state were then kept under constant conditions, in a CT room at 26±1°C, at about 70% relative humidity with a cycle of 12L:12D. New generations were set up regularly, i.e., at a 6- to 8-week interval. Each new generation was started with a randomly picked virgin female and virgin male. All bed bugs were maintained in the CT room with the conditions described as above. All individuals in our study were virgin prior to experiments. The feeding, maintenance and generation-of-virgin-individuals protocols follow Reinhardt *et al*. [327].

5.3.2 Segregation of BEV-like symbiont

Additionally, all isofemale lines were tested for the presence of the BEV-like symbionts. In the last chapter, I noted that previous studies have observed the presence of BEV-like symbionts as another facultative endosymbionts, but how they segregate has never been observed in bed bug populations [6, 347]. Presence in the R+ and R– lineages of F4 and S1 was therefore assessed, in order to establish that BEV-like symbiont and *Rickettsia* status did not covary (and therefore confound interpretation as to the source of differences).

The analysis of BEV-like symbiont presence was performed using a PCR assay. Two pairs of PCR primers were used in this test. The first pair was *BEVF and BEVR* (Table 4.3 in Chapter 4), which amplified a partial region of *16S rRNA* gene, based on the gene sequence of bacteria of the leaf hopper (*Euscelidius variegatus*) as described in Degnan *et al.* [321]. The second pair, CLBEVF and CLBEVR (Table 4.3) amplified segment of DNA gyrase subunit B (*gyrB*), designed in this study. The symbiont assays are the same as rickettsial screen described in Chapter 4 (PCR conditions are provided in the legend of Table 4.3). The amplicon products from the both genes were cleaned

with ExoSAP-IT kit (E1050, New England Biolabs, US) and Sanger sequenced to confirm the BEV-like symbiont strains.

5.3.3 Development time and sex ratio in a common garden experiment

A 7-day-old virgin male and female from each isofemale line were put together in a plastic pot and allowed to mate. Individuals from both sexes were fully fed as post-eclosion adults, and immediately prior to mating, which ensured gamete production until day 7. Once offspring hatched from the eggs laid, ten of 1st instar nymphs were collected from each pot (4 R+ and 4 R- sublines for each of S1 and F4, N = 160 total). Eight fresh plastic pots with filter paper and ventilation pores on the lids were prepared to house the nymphs. Ten *Rickettsia*-infected or ten *Rickettsia*-free nymphs were randomly put into each pot and presented with the opportunity to feed with human blood every three days. As soon as the first 5th instar nymph was observed in a pot, eclosion was checked every day. Freshly eclosed adults were then removed from the pot and *post hoc* screened for *Rickettsia* infection status using the PCR method described in Chapter 4 (PCR primers for *Rickettsia* in Table 4.3). The number of days between placement into the pot and the last hatching event, i.e., removal from the pot, represents the development time. The sexes of individuals were determined when the bugs reached the adult stage. Sex ratio (number of female:male) was calculated and compared between the two infection status, R+ and R- individuals, which were identified with the PCR assays.

5.3.4 Fecundity

To measure the effect of *Rickettsia* infection on fecundity, a full factorial crossing scheme of female x male were used (i.e., R+xR+, R+xR-, R-xR+ and R-xR-). For this, same-aged individuals (a 7-day-old virgin male and female) were put together in a pot and allowed to mate and feed weekly. Every week, all the eggs were collected and put in a fresh pot, which was fed weekly until 5th instar nymphs were observed. Same-aged 5th instars were then fed and placed into a 96-well plate until they reached adulthood. Seven-day-old virgin adults were then used in a full factorial crossing experiment. Prior to the experiment, females were fed twice, the last time on the day of mating, and males once, on the day of hatching. To avoid inbreeding

effects, each isofemale line was crossed with every other line, but not with itself. To have equal sample sizes for within versus between *Rickettsia*-free and *Rickettsia*-infected crosses, one cross was randomly left. Like this, each isofemale line was crossed with three *Rickettsia*-free lines and with three *Rickettsia*-infected lines (N = 96 crosses). Matings were staged, monitored and interrupted after 60s as described earlier in Reinhardt *et al.* [360]. Interrupted matings standardise sperm number because of the linear relationship between copulation duration and sperm number [361]. A standardised sperm number was desirable since spermatozoa trigger the release of an oviposition-stimulating hormone from the *corpora allata* [362] and could potentially influence lifespan through differential egg production. The use of 60s standard matings also allows comparability with other studies.

After mating, the females were kept individually in 15ml plastic tubes equipped with a piece of filter paper for egg laying. Females were fed weekly and the number of fertile and infertile eggs counted in weekly intervals. Fertile and infertile eggs were distinguished, and the onset of laying and fertilization senescence determined following Reinhardt *et al.* in 2009 [363]. The onset of infertility can be precisely obtained as the time point when the second infertile egg was laid, to allow for one accidental fertilization failure. Fertile eggs are taut and whitish with visible eye spots of the developing embryo. Infertile eggs normally collapse soon after being laid and are greyish. The number of fertile eggs was used to investigate the fecundity of crosses.

5.3.5 Cytoplasmic Incompatibility (CI)

To determine the occurrence of CI, the number of fertile eggs produced by females from different crossing combinations were observed [310]. When fertile eggs are laid, it implies that the embryos in the eggs have already passed the critical point of CI. It has been observed that about one-third of embryogenesis happens within the ovaries before the eggs are laid [278]. I expected that if *Rickettsia* induces CI in bed bugs, the proportion of fertile eggs will be lowest in the group in which only males from *Rickettsia*-infected line were crossed (R- x R+).

5.3.6 Body size

Mature adult males and females of R+ and R- lines from F4 and S1 populations were collected after the eclosion between 1 to 3 days old. At this point their cuticles have been sclerotized properly, allowing accurate body measurement. The width of the pronotum was selected as the criterion for body measurement (Figure 5.1). Measuring this character can precisely describe bed bug body size in which this character has highly positive corelation with other characters of body extremities [284]. Moreover, this hardened region is constant in shape and could be repeatedly measured without effects from the recent feeding [364]. Individual bed bugs were ventrally placed and adjusted posture on the reference grid which was immobilized by covering with a lightweight glass petri dish over the bug. Then its entire body field was photographed under a stereomicroscope (Leica M165C, Germany). The photographs were measured for the width of pronotum using FIJI program [365], a ruler tool was standardized by the reference scale of each image.

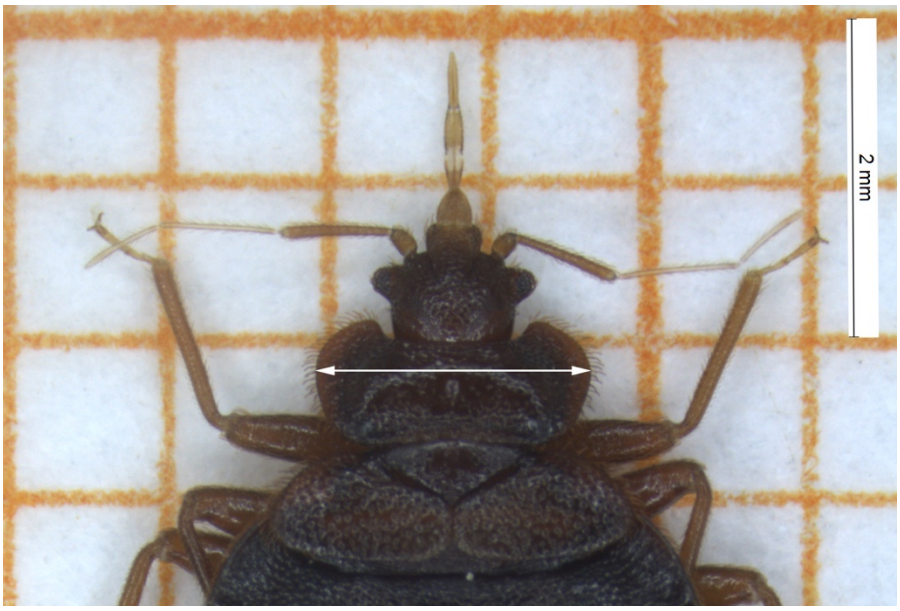

Figure 5.1 Pronotum width of the common bed bug. White arrow line indicates the width of pronotum, the character is used as the representative of *C. lectularius* body size in the effect of *Rickettsia* on *C. lectularius* body size.

5.3.7 Longevity

To observe the effect of *Rickettsia* on longevity of *C. lectularius*, the bed bugs were maintained at a raised temperature to shorten longevity to be tractable (lifespan at standard rearing can exceed a year). The temperature was set at 30^{o}C conditions. However, this temperature is not high enough to alter the titre of *Wolbachia* (the temperature that affected *Wolbachia* was observed at 36^{o}C [366]).

Adult *C. lectularius* (N= 24 for each male R+, male R-, female R+ and female from both F4 and S1, N= 192 total) were randomly selected to be the subjects for this experiment. Each bug was assigned to be in an individual well of 96-well plates (two plates mixed of infection lines and populations) with a small filter paper in the bottom of each well and a rubber lid with ventilation pores on the top. The date that a bug was placed in the well was marked for the start date. Observation was conducted over a 4-month-period. During the first 2 weeks, live/dead status was observed once a week and after that the observation was performed every day. From this, longevity was calculated.

5.3.8 Statistical analysis

Data were analysed using the statistical platform R (version 3.6.1, 2019) [259] under the packages 'lme4' [367] and 'survival' [368]. The analysis of development time, fecundity and body size measurement were done by fitting linear mixed-effects models (LMMs) using the 'lmer' function, while sex ratio and cytoplasmic incompatibility were fitted in generalised linear mixed effect model (GLMMs) using 'glmer' function with binomial family. The experiment on longevity was analysed using the Cox proportional hazards model [369] in 'coxph' function.

For the development time and sex ratio, I fitted 'pot' as a random effect of mixed effect models, while 'infection status' and bed bug 'populations' were fitted as the main effect in all cases. The factor 'sex' was also included as a fixed effect explaining development time. The ratio of 'female:male' and 'number of fertile:infertile eggs' were set as the response variable in the sex ratio and fecundity test, respectively.

In fecundity and CI analyses, ‘infection status’ was broken down into ‘male infection status’ and ‘female infection status’ as these two factors represented cross types (female x male; R+ x R+, R+ x R-, R- x R+, R- x R-), alongside the main factor ‘population’. Family of origin was modelled as a random effect.

The body size measurement, ‘pronotum width’ was fitted as the response variable. The ‘family of origin’ of individual bed bug was fitted as a random effect. Infection status, population and sex were fitted as fixed effects of LMMs.

For the longevity experiment, ‘survival probability’ was estimated using the Kaplan-Meier method in ‘survfit’ and ‘Surv’ function. The proportion of dead individuals was fitted as the response variable. Infection status, population and sex were fitted as independent factors in the Cox proportional hazards model.

Non-significant effects were removed from all models until the minimum adequate model was attained. Likelihood ratio tests (LRTs) were performed by comparing a null model with the minimum adequate models of LMMs, GLMMs and the Cox proportional hazards model using ‘anova’ function, considering χ^2 with critical *p*-value at 0.05. The normality and homoscedasticity of residuals of the LMMs was validated before the final interpretation as well as a validation of the proportional hazard assumptions.

5.4 RESULTS

5.4.1 Segregation of BEV-like symbiont

All the screened samples (R+ and R-) tested positive for BEV-like symbionts in the PCR assays. The alignment of sequences *16S rRNA* and *gyrB* genes suggested that only a single strain of the symbiont was found in both F4 and S1 populations.

5.4.2 Development of the instars and sex ratio

Development time showed a different pattern between the populations. In F4 the development times were similar across all four crosses (Figure 5.2). S1 showed a substantial difference between the development time of R+ and R- females, whereas R+ and R- males were the same. The full model analysis (including interaction terms between sex, population and *Rickettsia* infection status) was not a significantly better fit than the model without interaction terms with only infection factor (LRT $\chi 2(4) = 5.554$, p=0.235). The impact of individual terms was then examined in models without an interaction term. The only significant explanatory variable was 'infection' (LRT of models with and without infection term: $\chi 2(1) = 5.177$, $p = 0.023$). First instar-adult period for *Rickettsia*-infected individuals increased by 0.59±0.26 days (Mean±SD). The R+ bed bugs took 26.7±2.00 days to reach adulthood while the R- bugs took 25.9±2.15 days. The minimum adequate model of LMMs for development time analysis was:

development time ~ infection+(1|pot).

There was no impact of *Rickettsia* infection status on sex ratio of offspring (LRT: $\chi 2(1) = 0.0003$, $p = 0.985$) (Figure 6). Female: male ratio of the R- group was 0.84±0.29 and 0.93 ±0.67 for the R+ group. There was no interaction effect between infection status and population of origin (LRT: $\chi 2(1) = 0.078$, $p = 0.780$). The minimum adequate model of GLMMS for sex ratio analysis was:

cbind (number of females, number of males) ~ infection+(1|pot), error=binomial.

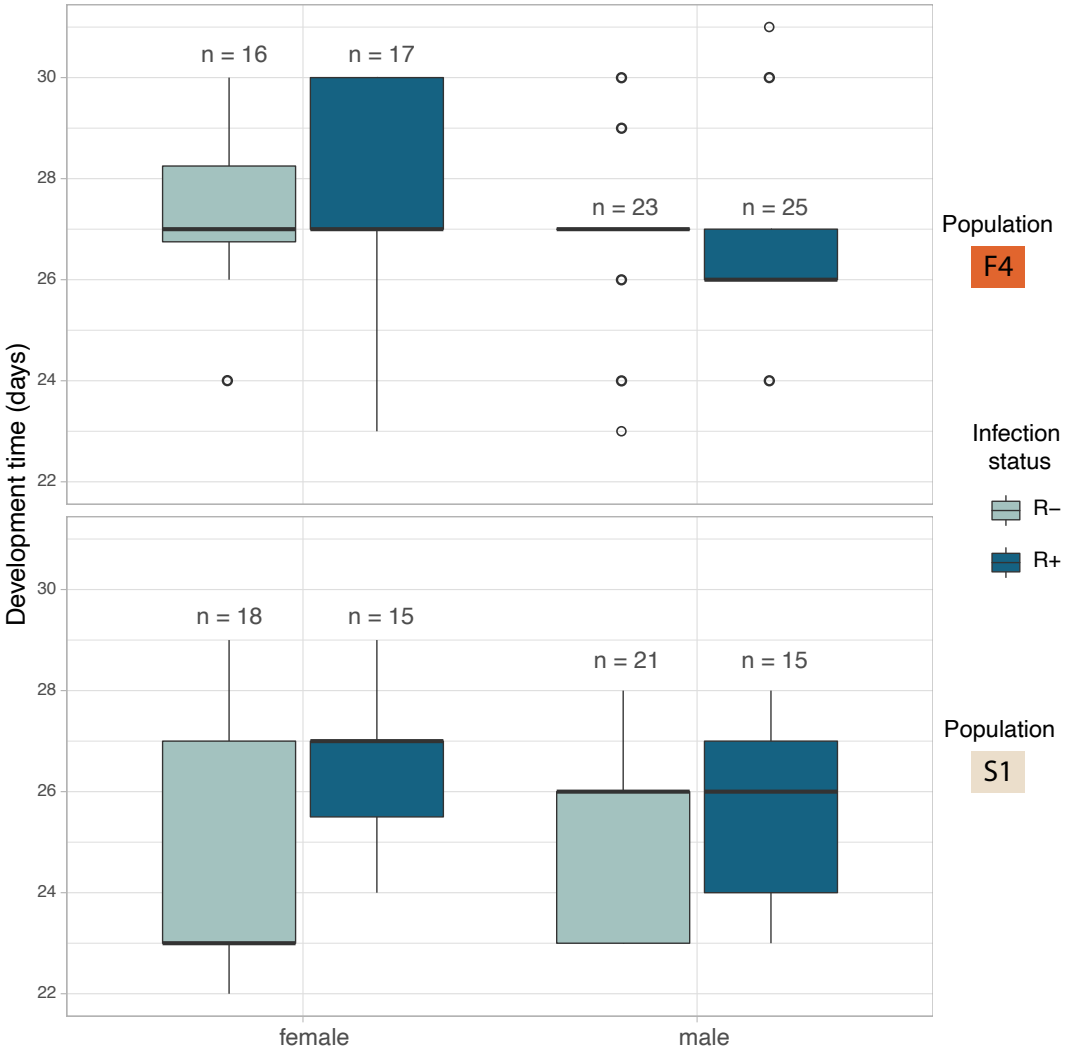

Figure 5.2 Median development time in days of *C. lectularius* from the first instar to adulthood for males and female individuals of *Rickettsia*-free (R-, light blue) and *Rickettsia*-infected (R+, dark blue) groups from population F4 (top) and S1 (below). *Rickettsia* infection has a significant effect on development time (LRT: $\chi 2(1) = 5.177$, $p = 0.023$). Boxes indicate the 25 and 75 percent quartiles respectively, the whiskers show minimum and maximum values. Open circles indicate potential outliers using the interquartile range (IQR) criterion, considered by R.

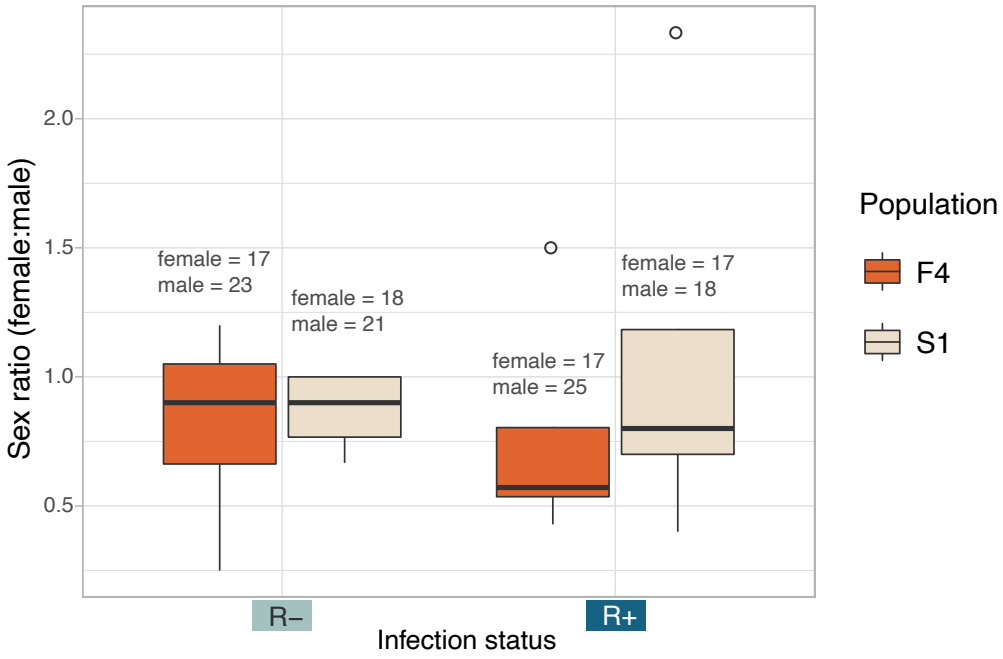

Figure 5.3 Median sex ratio (number of female:male) of the *Rickettsia*-free and *Rickettsia*-infected *C. lectularius* adults from the two populations. The sexes were identified from adult bed bugs. There was no significant difference in the sex ratio between R- and R+ group at $p = 0.05$. Total number of males and females are shown above the boxes. Boxes indicate the 25 and 75 percent quartiles respectively, the whiskers show minimum and maximum values. Open circles indicate potential outliers using the interquartile range (IQR) criterion, considered by R.

5.4.3 Fecundity and CI

The analysis of fecundity (total fertile eggs) by Likelihood ratio comparison of LMMs indicated that there was no evidence of an interaction effect between the three factors, i.e., population, male and female infection status (LRT: $\chi 2(3) = 5.781$, $p = 0.216$), and these terms were dropped from the model. The final model detected *Rickettsia* infection in the female parent as the sole significant explanatory variable for fecundity (LRT: $\chi 2(1) = 4.576$, $p = 0.032$). Infected females were likely to produce fewer fertile eggs (R+ x R+ = 86.80 $\pm$ 34.30, R+ x R- = 89.70 $\pm$ 28.30) compared to uninfected females (R- x R+ = 107.00 $\pm$ 34.90, R- x R- 109.00 $\pm$ 34.60, Figure 5.4 A). The minimum adequate model of LMMs for fecundity analysis was:

fertile eggs ~ female infection status+(1|female family of origin/population) + (1|male family of origin/population).

The relative ratio of fertile:infertile eggs was analysed in GLMMs to ascertain if there was any evidence of cytoplasmic incompatibility. The likelihood ratio comparisons indicated no evidence of heterogeneity associated with *Rickettsia* infection in either male (LRT: $\chi 2(1) = 0.593$, $p = 0.441$) or female parents (LRT: $\chi 2(1) = 1.174$, $p = 0.279$). There was no evidence of an interaction term between male infection x female, evidenced by the statistical equivalence of models with and without an interaction term (LRT: $\chi 2(4) = 6.725$, $p = 0.151$, Figure 5.4 B). The minimum adequate model of LMMs for CI analysis was:

Cbind (fertile eggs, infertile eggs) ~ female infection status + male infection status + (1|female family of origin/population) + (1|male family of origin/population).

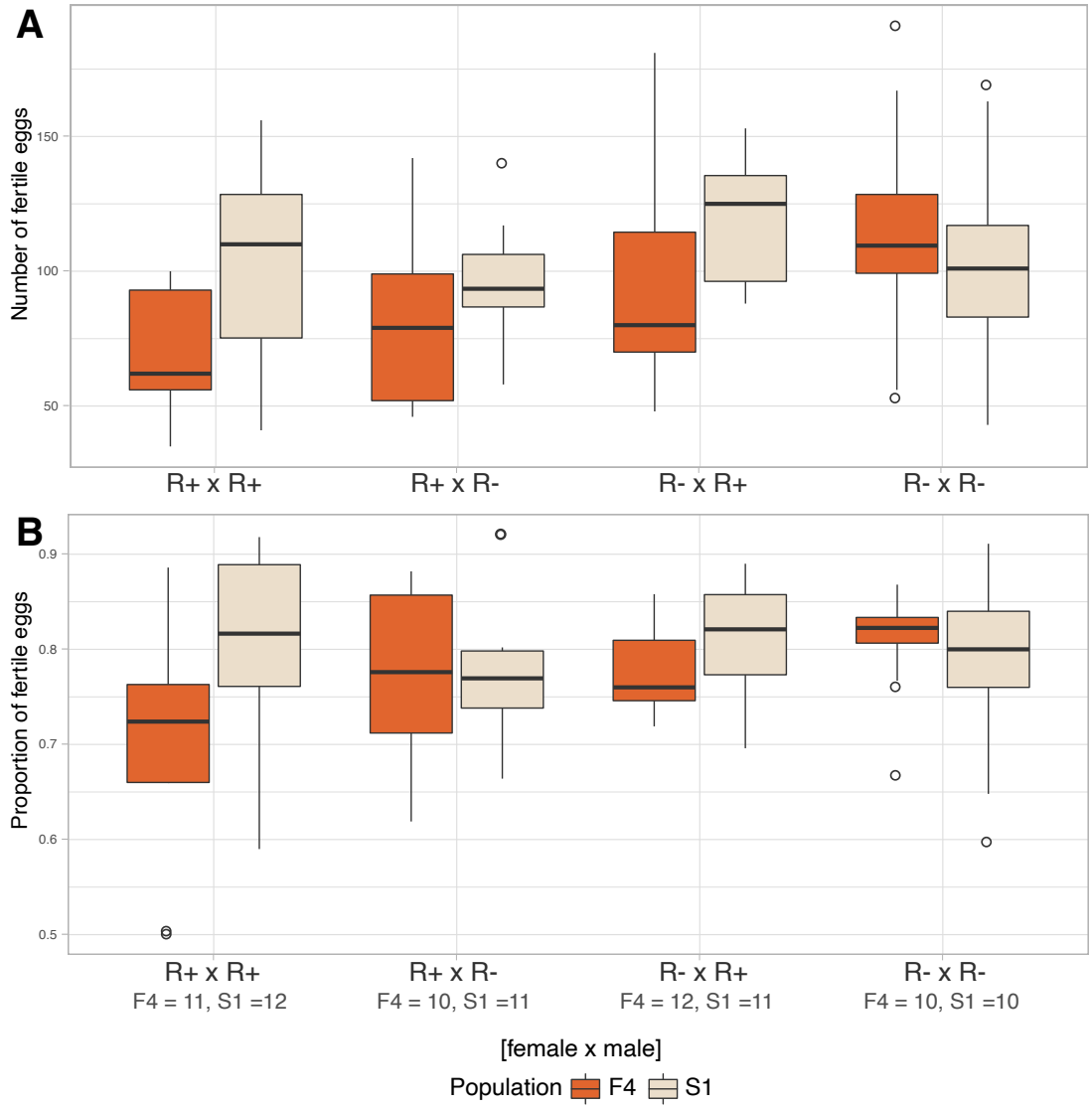

Figure 5.4 Fecundity and CI. **A**: Median number of fertile eggs of *C. lectularius* from population F4 and S1 from the four cross combinations. *Rickettsia*-infected female crosses (R+ x R+ and R+ x R-) produced significantly fewer fertile eggs when compare to other crosses (LRT: $\chi 2(1) = 4.576$, $p = 0.032$). **B**: Median proportion of fertile eggs of *C. lectularius* from the two populations. There was no interaction effect of male and female infections on the ratio of fertile:infertile eggs from the GLMMs analysis at $p = 0.05$, indicating there was no evidence of CI. Number of crosses completed are shown under the cross group. Boxes indicate the 25 and 75 percent quartiles respectively, the whiskers show minimum and maximum values. Open circles indicate potential outliers using the interquartile range (IQR) criterion, considered by R.

5.4.4 Body size

The body size of *C. lectularius* was estimated by the width of pronotum of the bugs. The pronotum width were fitted in LMMs as the dependent variable. There was no evidence of an interaction effect between the three factors, i.e., population, sex and infection status (LRT: $\chi 2(4) = 5.4178$, $p = 0.2422$). Only sex had a significant influence on body size of *C. lectularius* (LRT: $\chi 2(1) = 157.9700$, $p < 0.001$). In both populations, females have a wider pronotum (1.67 ± 0.07 mm for F4 and 1.62 ± 0.07 mm for S1) than males (1.57 ± 0.08 mm for F4 and 1.51 ± 0.05 for S1; Figure 5.5). Infection with *Rickettsia* and population did not have a significant effect on body size of *C. lectularius* (LRT: $\chi 2(1) = 2.2803$, $p = 0.131$ and $\chi 2(1) = 2.108$, $p < 0.1465$, respectively). The minimum adequate model of LMMs for body size analysis was:
pronotum width ~ sex + (1|family of origin).

5.4.5 Longevity

Likelihood ratio test of the Cox proportional hazards models indicated that there was no interaction effect between infection status, sex and population (LRT: $\chi 2(4) = 8.5028$, $p = 0.0748$). The longevity of *C. lectularius* are not affected by *Rickettsia* infection (LRT: $\chi 2(1) = 2.535$, $p < 0.111$) and population (LRT: $\chi 2(1) = 2.051$, $p < 0.1521$) in 30^{o}C rearing condition. Only the sex of the bed bugs had a significant effect on longevity at 30^{o}C (LRT: $\chi 2(2) = 51.555$, $p < 0.001$; Figure 5.6). Female bed bugs had longer longevity (78.2 ± 2.4 days for F4 and 79.2 ± 2.2 for S1) than males (62.1 ± 1.9 days for F4 and 64.8 ± 2.0 days for S1). The minimum adequate model of Cox proportional hazards model for longevity analysis was:
Surv (days, survival status) ~ sex.

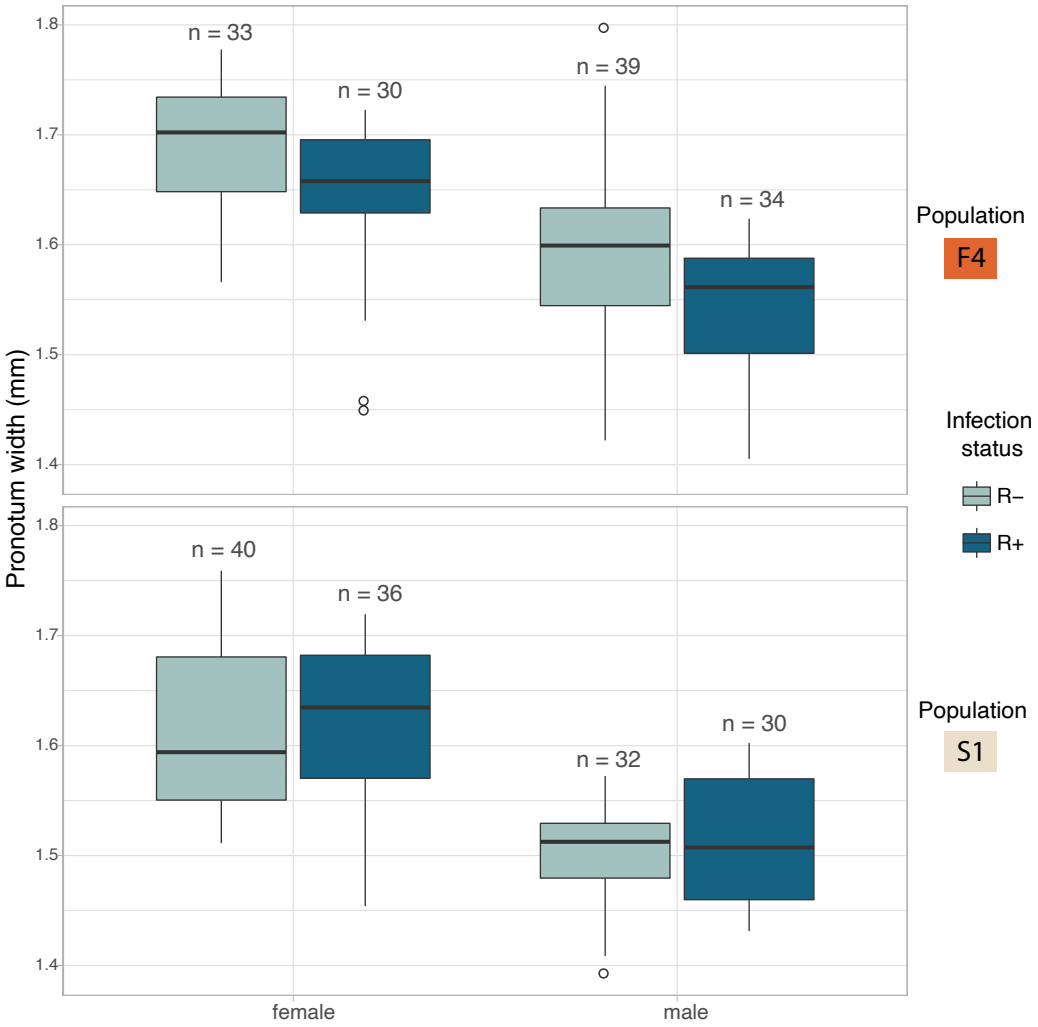

Figure 5.5 Body size of *C. lectularius* of different sex and *Rickettsia* infection status. The body size was estimated from the width of pronotum (in millimetre) of the male and female adults in *Rickettsia* infected (R+) and *Rickettsia*-free (R-) lines from populations F4 (top) and S1 (below). Likelihood ratio of linear mixed effect models indicated no evidence that *Rickettsia* infection affected the width of pronotum (LRT: $\chi 2(1) = 2.2803$, $p = 0.131$). Boxes indicate the 25 and 75 percent quartiles respectively, the whiskers show minimum and maximum values. Open circles indicate potential outliers using the interquartile range (IQR) criterion, considered by R.

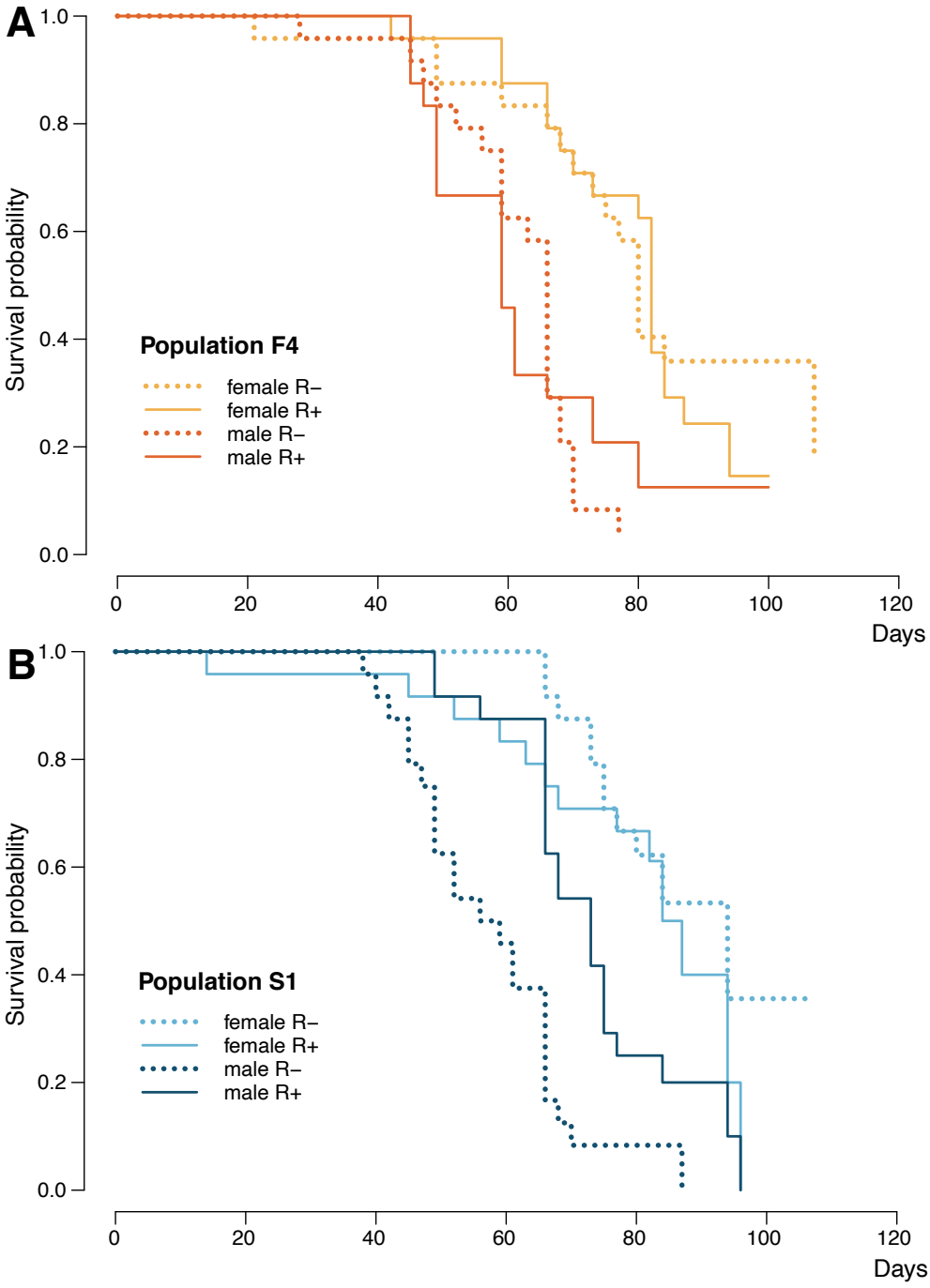

Figure 5.6 Kaplan-Meier survival curve of *C. lectularius*. The survival curves estimate longevity of male and female adults *C. lectularius* in *Rickettsia*-infected (R+) and *Rickettsia*-free (R-) lines from F4 (**A**) and S1 (**B**) populations. Likelihood ratio of the Cox hazard models indicated that *Rickettsia* infection does not affect longevity of *C. lectularius* in 30°C (LRT: $\chi 2(1) = 2.535$, $p < 0.111$). Only sex has a significant effect on longevity of bed bugs at 30°C (LRT: $\chi 2(1) = 51.555$, $p < 0.001$). The curves illustrate that females (lighter colour lines) have longer longevity than males (darker colour lines) in both populations.

5.5 DISCUSSION

Maternally inherited endosymbionts are likely to have impacts on their host biology. Controlled experimental analysis of the impact of torix *Rickettsia* on their host have not been previously completed. In this study, I utilised the presence of infected/uninfected *Rickettsia* lines of two bedbug populations to determine the influence of *Rickettsia* on the host in terms of both reproductive parasitism (sex ratio distortion, CI) and a variety of life history parameters (development rate, adult body size, fecundity and longevity). These experiments revealed no evidence of positive effects on host performance, no evidence of reproductive parasitism, but slightly pathogenic effects of *Rickettsia* carriage.

5.5.1 Impact of other symbionts

One caveat to these results is that they are performed on two particular genetic backgrounds, rather than across a broad range of backgrounds. Both backgrounds harboured the BEV-like symbiont. Thus, the conclusions made are specific to this background. It is possible, and important to investigate, if the results are distinct when the BEV-like symbiont is not present.

It is typical that when different strains of symbiont co-exist there may be an interaction among them, either direct or indirect [345]. The presence of other symbionts can certainly alter the titre of focal strains, and likely also modify the biological impacts [110, 141, 370]. However, curing the BEV-like symbiont is not straightforward, as any intervention must not impact *Wolbachia*, the required symbiont [6]. It may be possible to transinfect *Rickettsia* instead or find a line where *Rickettsia* has segregated (as in F4/S1) but the BEV-like symbiont is not present.

The experiments either demonstrated no detectable effect of the *Rickettsia*, or mild deleterious impacts. The nature of this seems compatible with a low physiological cost to carrying *Rickettsia*, which may be either direct metabolic activity, or through interference with host systems, or with *Wolbachi*a function. The *Rickettsi*a is not overtly pathological but does impose a cost to the host under laboratory conditions. The results are similar to those observed in the *Spalangia*-

Rickettsia interaction, where *Rickettsia* (again in the presence of *Wolbachia*) was associated with a one-day developmental delay, but did not induce changes in either sex ratio or CI [110].

5.5.2 Consequence of *Rickettsia* segregation

These data indicate that we are missing important aspects of the symbiosis. In Chapter 4, it was noted that *Rickettsia* infection passed with very high fidelity from mother to offspring in the laboratory crosses, but that segregation has occurred over the ten years between 2006 and 2018 in the Sheffield laboratory. If segregation occurs, it is expected that the *Rickettsia* would progressively be lost in the absence of beneficial effects or reproductive parasitism. The symbionts are generally selected to be retained within the host matriline if they have a role on host reproductions or conferring an advantage to host fitness [371].

However, these experiments found no evidence for beneficial effects. This contrast makes it likely that subtle beneficial effects do exist but are either too small to be detected in this study, or are ecologically dependent – they occur but not in the confines of the laboratory. Resistance to xenobiotics (e.g., insecticides), thermal sensitivity, resistance to natural enemies (e.g., bacterial, viral pathogens) have all been observed in other studies of symbiosis [124-126, 155, 157, 372] and should be tested in this one.

5.6 CONCLUSION

The segregation of torix *Rickettsia* within *Cimex lectularius* laboratory populations from the previous chapter were used to examine the biological impacts of symbiosis in this study. Under the laboratory conditions, bed bugs with *Rickettsia*-infected (R+) developed significantly slower and produced fewer fertile eggs than the *Rickettsia*-free line (R-). This negative effect seemed to reduce benefits for both male and female hosts and so reflects a mild parasitic relationship. However, no reproductive parasitic phenotypes were observed, with both sex-ratio distortion and cytoplasmic incompatibility being excluded. Finally, these results are representing only a few perspectives of torix *Rickettsia* in *C. lectularius* host system. Further studies on how torix *Rickettsia* alter host benefits (e.g., providing xenobiotic resistance and protections) and observing them through their genomic structure are worth exploring as well as other dimensions of the symbiosis.

CHAPTER 6

Thesis summary

6.1 OVERVIEW

Since the discovery of non-vertebrate pathogenic *Rickettsia* in 1994, *Rickettsia* have been increasingly recognised as a clade of arthropod-associated symbiont, which play an important role in host biology. In 2002, a new clade of *Rickettsia* – the torix group – was first described from leeches. Since this time, torix group *Rickettsia* have been detected in a broad range of eukaryotic taxa, especially in freshwater-associated groups, which led to first an investigation of their presence in Odonates, and second, testing the hypothesis that freshwater taxa represent a hot-spot for infection. Importantly, despite the building evidence that torix *Rickettsia* were common invertebrate symbionts, the biological impact on the host is very poorly characterised. The thesis addressed this issue through developing a *Rickettsia*-bed bug system for study.

Consistent with the aquatic hot-spot hypothesis, I observed torix *Rickettsia* in Odonata with diverse strains that found across dragonfly and several damselfly species over three geographic regions. Damselflies in the family Coenagrionidae were observed as a hot-spot for this symbiont. FISH imaging indicated torix *Rickettsia* were present in ovary tissue of the azure damselfly (*Coenagrion puella*), and thus likely inherited via a vertically transmitted route. Sharing of symbiont strains between the sibling species *Coenagrion puella* and *C. pulchellum* indicated that *Rickettsia* has likely driven mtDNA introgression between the species and represents a further symbiont that can disrupt mtDNA barcoding studies.

Following this, the freshwater hot-spot hypothesis was tested. Insect orders from freshwater communities were more commonly associated with torix *Rickettsia* than terrestrial samples. The study additionally indicated a number of novel taxa for torix *Rickettsia*. Most significant here is the finding of a *Rickettsia* symbiosis in a Gastropod mollusc, an animal group with few previous records of symbiosis. Additionally, the screen revealed a second potential hot-spot in blood-feeding insects, including mosquitoes, black flies and bed bugs. The medical and veterinary significance of these host species, combined with the potential impact of symbiosis

on vector competence, makes these an interesting set of symbioses for onward study.

One of the species highlighted as a host to torix *Rickettsia* was the nuisance human ectoparasite, the common bed bug (*Cimex lectularius*). Bed bugs and one of their allied species, the African bat bug (*Afrocimex contrictus*) were observed to carry a potential identical torix *Rickettsia* strain, based on the *16S rRNA* and *gltA* sequences. The *C. lectularius* system was developed for onward study. Maternal inheritance was observed to be the only transmission route for this symbiont, with lack of paternal inheritance contrasting with the only previous study of inheritance patterns. The infection localised in oocytes and bacteriomes, the former consistent with successful maternal inheritance, and the latter providing an arena for interaction with the obligate symbiont *Wolbachia*, as well as permitting the use of *Wolbachia* transmission mechanisms – involving bacteriocyte movement – to establish in the ovary.

I then used the segregation of *Rickettsia* within bed bug laboratory populations to examine the biological basis of symbiosis. Bed bugs with *Rickettsia*-infected (R+) developed significantly slower and produced fewer fertile eggs than the *Rickettsia*-free line (R-). These finding suggested the form of their facultative relationship is a mild parasitism. However, no reproductive parasitic phenotypes were observed, with both sex-ratio distortion and cytoplasmic incompatibility being excluded.

To summarise:

i) Torix *Rickettsia* are common symbiotic partners of invertebrates, infecting diverse invertebrate taxa which now includes gastropods and Odonates.

ii) These symbioses likely drive mtDNA introgression, and thus join *Wolbachia* as a factor that may disrupt barcoding studies.

iii) PCR screening data supports the aquatic hot-spot hypothesis within insects.

iv) There is evidence of *Rickettsia* infection in many haematophagous species, including pest and vector species.

v) The bed bug system represents a useful model for investigating biological impacts, as it has segregating lines that are isogenic apart from *Rickettsia*, and can be kept in the laboratory.

vi) The *Rickettsia* within bed bugs are not reproductive parasites, but a positive phenotypic effect could not be determined.

6.2 FUTURE DIRECTION

6.2.1 Why is the freshwater biome a hot-spot for torix *Rickettsia* infection?

Even though this study revealed statistical evidence that freshwater-associated insects are a hot-spot for torix *Rickettsia,* the drivers of this pattern are not clear. It could be that the common water medium supports the movement of this endosymbiont. However, water alone cannot maintain the survival of these bacteria as they are obligate intracellular symbionts. Thus, microeukaryotic hosts that are common in water possibly play an important role in the movement of torix *Rickettsia*, and unusually torix *Rickettsia* are known to infect both microeukaryotes and invertebrates. In order to explain this pattern, I will propose two possible models based on the distribution pattern of endosymbionts from microeukaryotes.

Micro-eukaryotic Source Model:

Castelli *et al*. in 2016 [192] infer the ancestral Rickettsiaceae are generally retrieved from microeukaryotes. Indeed, the majority of non-*Rickettsia* members are known solely from microeukaryotes (*Trichorickettsia*, *Gigarickettsia*, *Phycorickettsia*), or are strongly biased to these taxa (*Candidatus* Megaira). An implication of this is that horizontal transmission of these bacteria can frequently occur among phylogenetically distant host species [270, 373]. If we include torix *Rickettsia* within this, then the aquatic environment provides a rich source of potential symbiotic partners as well as an avenue for symbiont movement.

The *Rickettsia* relative, *Candidatus* Megaira (previously Hydra group of *Rickettsia*), emerged roughly 220 mya before the emergence of torix clade (Figure 1.3 in Chapter 1). This bacterium is a major endosymbiont in microeukaryotes living in both oceans and freshwaters, e.g., green algae [374-376], amoebae [377], ciliates [378, 379], and cnidarians [380, 381]. These organisms are primitive in body plan and are important in many trophic chains [382, 383]. The presence of related strains in evolutionary disparate host species make it very likely that *Candidatus* Megaira are horizontally transmitted within the aquatic ecosystem [270, 384], and by extension

this supports a similar route for torix *Rickettsia* of microeukaryotes transmitting into freshwater invertebrate species at higher trophic levels (Figure 6.1 A).

Against the hypothesis of microeukaryote involvement are the relative rarity of torix strains in microeukaryotes, one in the amoeba *Nuclearia pattersoni* [164], the other in *Pompholyxophrys* [269]. Are these rare records a true indication that torix *Rickettsia* are uncommon in microeukaryotes, or is there a bias against finding them from lack of screening or other focal studies? Screening for torix *Rickettsia* in the broad spectrum of those primitive micro-eukaryotes would give more resolution on distribution patterns of torix *Rickettsia* and the relatedness among these strains will help to understand the movement and potential emerging point of torix clade.

Invertebrate Circulation Model:

An alternate theory is that torix *Rickettsia* were introduced to aquatic invertebrates millions of years ago, and their incidence in this group is associated with horizontal transfer amongst invertebrate members of the community (Figure 6.1 B). Here, torix *Rickettsia* may have established initially from microeukaryotic hosts, but the spread within the community is largely from host shifts from one invertebrate species to another. For instance, shared ectoparasites and predators may enable the horizontal distribution among multicellular taxa.

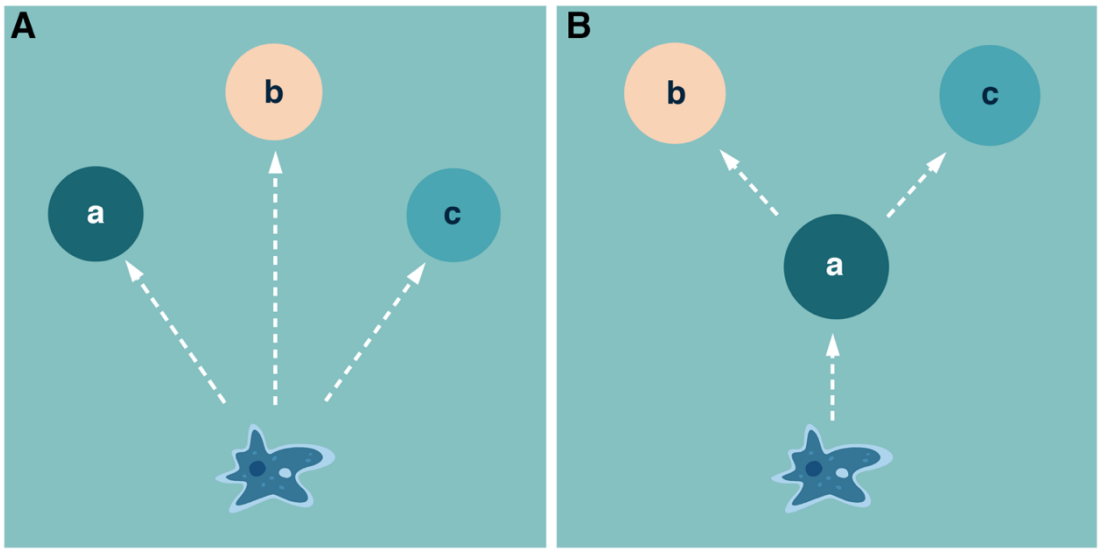

Figure 6.1 Transmission theory of torix *Rickettsia*. **A**: Micro-eukaryotic Source Model has the infection horizontally spreading (dash arrow) from amoeba to other advanced eukaryotes, species 'a', 'b' and 'c'. **B**: Illustration indicates Invertebrate Circulation Model by the first infection horizontal spreading from amoeba to advanced eukaryote species 'a'. Then the infected species 'a' transmit the infection to species 'b' and 'c' via transfer between invertebrate species.

6.2.2 Why do torix *Rickettsia* have such a broad host range?

Non-torix *Rickettsia* are found in arthropods [144, 150, 182], in contrast to *Megaira* that is hosted by a diverse range of aquatic microeukaryotes [270]. However, torix *Rickettsia* hosts cover a broad-spectrum of microeukaryotes (i.e., amoebae) to non-arthropod (mollusc and leech) and arthropod taxa. The factors that drive these endosymbiont transitions from microeukaryote to multicellular taxa is unclear. A key feature required to persist in multicellular taxa with a differentiated germline is the capacity to locate and invade this tissue, a tropism not required in host species with fission or other simpler forms of reproduction (budding). Even in simple multicellular taxa, such as *Volvox*, *Megaira* simply infects all cells including the germ line [375]. Thus, evolution to utilize invertebrates likely involves germ line targeting mechanisms. This, however, does not explain why other *Rickettsia* are restricted to arthropods.

It may be possible to examine the transitions through an in-depth comparative genomic study of torix and their relatives Rickettsiaceae, other arthropod-associated *Rickettsia* clades and *Megaira*. A previous genomic study of torix *Rickettsia* was performed for the strain associated with biting midges (*Culicoides newsteadi*) [175]. The draft genome revealed a complete gene family of the Pentose Phosphate Pathway (PPP). The PPP helps to maintain carbon homeostasis to retain a function in glycolysis and to reduce oxidative stress [385]. This gene family is functional in some parasites that need the PPP to reduce oxidative stress when they invade the host [386]. Notably, this is a common property of torix *Rickettsia* and *Megaira* [175]. However, some features of this gene family are absent in other arthropod-associated *Rickettsia* suggested it was independently lost during the transition to be an arthropod endosymbiont [175]. This function may help torix *Rickettsia* succeed in infecting a wide range of eukaryotic host taxa. Further genomic investigations in other torix *Rickettsia* strains, and indeed *Megaira*, may help to understand features enabling a broad host range in this clade.

6.2.3 From aquatic to the land

The emergence of the torix clade still needs further investigation to indicate whether their origin is from aquatic or terrestrial ecosystems, even though the theory of horizontal transmission from aquatic-microeukaryotes is likely. An extension of this is how torix *Rickettsia* emerged from aquatic microeukaryote-associated Rickettsiaceae to invade the terrestrial biomes. Three scenarios might be considered (Figure 6.2).

1.) The transition occurred when aquatic macroeukaryote species horizontally transmit to terrestrial hosts via predation or parasitism. The species that have both aquatic and terrestrial phases might be involved in the infection spreading onto the land species, e.g., trichopterans and odonates. These will commonly be consumed by spiders living near water.

2.) The transition occurred when aquatic species evolved a terrestrial lifestyle, thus the terrestrial taxa acquire infection through vertical transmission. Whilst this may have occurred historically, the diverse range of terrestrial hosts [254] indicates that vertical transmission cannot account for most cases.

3.) The transition occurred when aquatic microeukaryotes horizontally transmit to terrestrial eukaryotes via predation or parasitism. This may be hypothesised that the terrestrial hosts accidentally acquired aquatic micro-eukaryotes carrying torix *Rickettsia*, for instance, through drinking water and decomposing detritus [387].

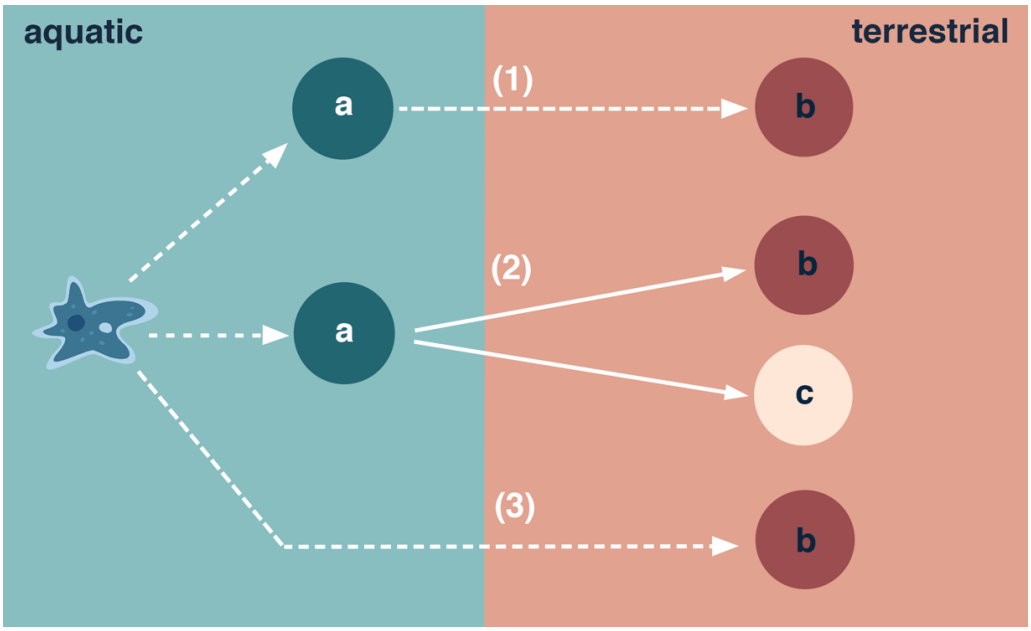

Figure 6.2 Transition patterns of torix *Rickettsia* from aquatic associated host to terrestrial host species. (1) The transition occurred when aquatic macro-eukaryote species horizontally transmit (dash line) to terrestrial eukaryotes via predation or parasitism. (2) The transition occurred when aquatic macro-eukaryote species vertically transmit to terrestrial eukaryotes via speciation (solid lines). (3) The transition occurred when aquatic micro-eukaryotes horizontally transmit to terrestrial eukaryotes via predation or parasitism.

6.2.4 Biological impacts of torix *Rickettsia*

Many studies have reported potential hosts for torix *Rickettsia,* but few have examined the impact of this endosymbiont infection on their host individual. This thesis demonstrated basic features of torix *Rickettsia* carriage based on the studies of two insect host systems, damselflies and the common bed bug. Overall, both cases revealed impacts that are negative.

In the bed bug, expression of mild parasitic impacts was observed: The results revealed that torix *Rickettsia* slowed development time and reduced fecundity. Even though these impacts are not directly lethal to the insects, they significantly decrease the certain fitness parameters and potentially affect other biological aspects of the insect.

However, torix *Rickettsia* infection have also been reported for positive effects in other host-symbiont systems. In the case of glossiphoniid leeches [135], torix *Rickettsia* could have positive impacts on the host leeches: they are associated with increasing body size. Whilst the mechanism and consequence of enlarging the body is unknown, this phenotype is known to be associated with improved leech reproduction [388].

Finally, in the bed bug system, it is likely that there are 'hidden benefits'. The symbiont does show vertical transmission loss at a low rate and does not exhibit reproductive parasitism. Thus, if the symbiont is to be maintained, there must either be a low level of infectious transfer, or there must be a positive fitness on bed bug reproduction. Future research on this system in immune regulations and protection roles of this endosymbionts (e.g., resistance to xenobiotics and protective against pathogens) may help to understand whether toix *Rickettsia* confer an ecologically contingent benefit to their bed bug hosts.

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APPENDIX

Appendix Table S1 SNP sites across MLST genes from ten *Coenagrion puella* and three *C. pulchellum* individuals, with non-synonymous substitution sites marked in bold. Non-synonymous/synonymous substitutions of each gene are in the bracket.

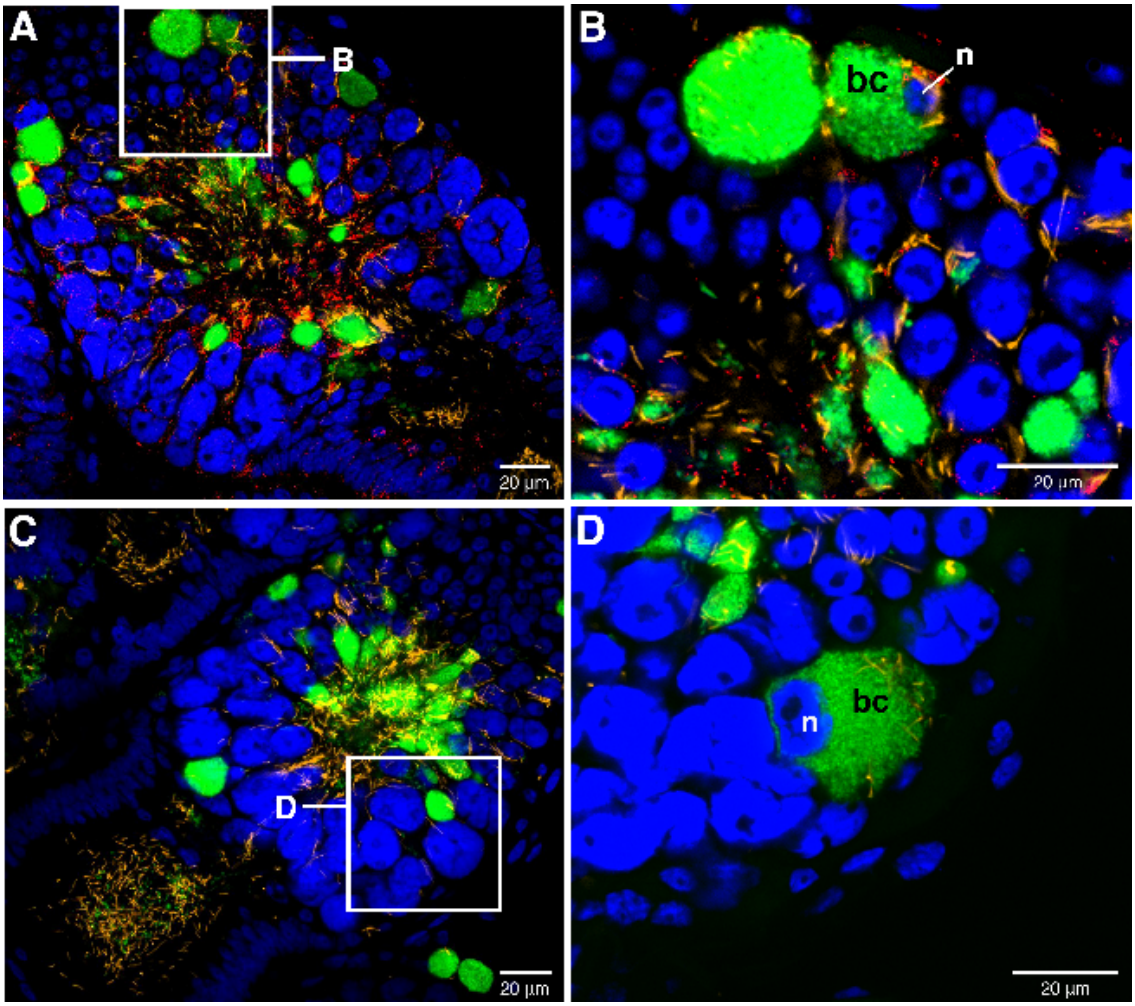
atpA **660 bps (12/38)**																	
ID	Variable sites																
	4	16	25	28	34	37	**43**	**59**	**65**	**68**	70	**75**	**79**	91	94	97	106
C. puella																	
1	T	T	C	G	A	A	**G**	**T**	**C**	**A**	T	**A**	**C**	G	T	C	T
2	C/T	C/T	C/T	A/G	A/C	A/G	**C/G**	**G/T**	**A/C**	**A/G**	C/T	**A/C**	**C/G**	A/G	C/T	C/T	C/T
3	T	T	C	G	A	A	**G**	**T**	**C**	**A**	T	**A**	**C**	G	T	C	T
4	C/T	C/T	C/T	A/G	A/C	A/G	**C/G**	**G/T**	**A/C**	**A/G**	C/T	**A/C**	**C/G**	A/G	C/T	C/T	C/T
5	C/T	C/T	C/T	A/G	A/C	A/G	**C/G**	**G/T**	**A/C**	**A/G**	C/T	**A/C**	**C/G**	A/G	C/T	C/T	C/T
6	T	T	C	G	A	A	**G**	**T**	**C**	**A**	T	**A**	**C**	G	T	C	T
7	T	T	C	G	A	A	**G**	**T**	**C**	**A**	T	**A**	**C**	G	T	C	T
8	T	T	C	G	A	A	**G**	**T**	**C**	**A**	T	**A**	**C**	G	T	C	T
9	C/T	C/T	C/T	A/G	A/C	A/G	**C/G**	**G/T**	**A/C**	**A/G**	C/T	**A/C**	**C/G**	A/G	C/T	C/T	C/T
10	C/T	C/T	C/T	A/G	A/C	A/G	**C/G**	**G/T**	**A/C**	**A/G**	C/T	**A/C**	**C/G**	A/G	C/T	C/T	C/T
C. pulchellum																	
1	T	T	C	G	A	A	**G**	**T**	**C**	**A**	T	**A**	**C**	G	T	C	T
2	C/T	C/T	C/T	A/G	A/C	A/G	**C/G**	**G/T**	**A/C**	**A/G**	C/T	**A/C**	**C/G**	A/G	C/T	C/T	C/T
3	C/T	C/T	C/T	A/G	A/C	A/G	**C/G**	**G/T**	**A/C**	**A/G**	C/T	**A/C**	**C/G**	A/G	C/T	C/T	C/T
ID	109	112	128	130	137	175	184	223	**224**	**225**	**226**	250	**276**	**277**	**284**	289	307
C. puella																	
1	T	C	A	A	C	G	C	T	**G**	**G**	**T**	T	**G**	**G**	**G**	A	G
2	C/T	C/T	A/C	A/G	A/C	G/T	C/T	C/T	**A/G**	**A/G**	**C/T**	C/T	**A/G**	**A/G**	**A/G**	A/T	A/G
3	T	C	A	A	C	G	C	T	**G**	**G**	**T**	T	**G**	**G**	**G**	A	G
4	C/T	C/T	A/C	A/G	A/C	G/T	C/T	C/T	**A/G**	**A/G**	**C/T**	C/T	**A/G**	**A/G**	**A/G**	A/T	A/G
5	C/T	C/T	A/C	A/G	A/C	G/T	C/T	C/T	**A/G**	**A/G**	**C/T**	C/T	**A/G**	**A/G**	**A/G**	A/T	A/G
6	T	C	A	A	C	G	C	T	**G**	**G**	**T**	T	**G**	**G**	**G**	A	G
7	T	C	A	A	C	G	C	T	**G**	**G**	**T**	T	**G**	**G**	**G**	A	G
8	T	C	A	A	C	G	C	T	**G**	**G**	**T**	T	**G**	**G**	**G**	A	G
9	C/T	C/T	A/C	A/G	A/C	G/T	C/T	C/T	**A/G**	**A/G**	**C/T**	C/T	**A/G**	**A/G**	**A/G**	A/T	A/G
10	C/T	C/T	A/C	A/G	A/C	G/T	C/T	C/T	**A/G**	**A/G**	**C/T**	C/T	**A/G**	**A/G**	**A/G**	A/T	A/G
C. pulchellum																	
1	T	C	A	A	C	G	C	T	**G**	**G**	**T**	T	**G**	**G**	**G**	A	G
2	C/T	C/T	A/C	A/G	A/C	G/T	C/T	C/T	**A/G**	**A/G**	**C/T**	C/T	**A/G**	**A/G**	**A/G**	A/T	A/G
3	C/T	C/T	A/C	A/G	A/C	G/T	C/T	C/T	**A/G**	**A/G**	**C/T**	C/T	**A/G**	**A/G**	**A/G**	A/T	A/G

ID	319	322	343	346	349	373	397	406	409	418	430	440	445	517	601	652
C. puella																
1	G	T	T	A	C	G	C	T	T	T	C	T	T	G	G	T
2	A/G	A/T	A/T	A/C	C/T	A/G	C/T	A/T	C/T	C/T	C/T	C/T	G/T	A/G	G/T	C/T
3	G	T	T	A	C	G	C	T	T	T	C	T	T	G	G	T
4	A/G	A/T	A/T	A/C	C/T	A/G	C/T	A/T	C/T	C/T	C/T	C/T	G/T	A/G	G/T	C/T
5	A/G	A/T	A/T	A/C	C/T	A/G	C/T	A/T	C/T	C/T	C/T	C/T	G/T	A/G	G/T	C/T
6	G	T	T	A	C	G	C	T	T	T	T	T	T	G	G	T
7	G	T	T	A	C	G	C	T	T	T	T	T	T	G	G	T
8	G	T	T	A	C	G	C	T	T	T	T	T	T	G	G	T
9	A/G	A/T	A/T	A/C	C/T	A/G	C/T	A/T	C/T	C/T	C/T	C/T	G/T	A/G	G/T	C/T
10	A/G	A/T	A/T	A/C	C/T	A/G	C/T	A/T	C/T	C/T	C/T	C/T	G/T	A/G	G/T	C/T
C. pulchellum																
1	G	T	T	A	C	G	C	T	T	T	T	T	T	G	G	T
2	A/G	A/T	A/T	A/C	C/T	A/G	C/T	A/T	C/T	C/T	C/T	C/T	G/T	A/G	G/T	C/T
3	A/G	A/T	A/T	A/C	C/T	A/G	C/T	A/T	C/T	C/T	C/T	C/T	G/T	A/G	G/T	C/T

	16S	***gltA***																
	1029 bp	**715 bp (2/14)**																
ID		Variable sites																
	15	27	33	**40**	**74**	123	129	130	192	216	228	234	246	296	390	444	453	
C. puella																		
1	A	A	A	**T**	**C**	T	T	C	G	A	C	T	C	T	G	T	T	
2	A/G	A/G	A/G	**G/T**	**C/T**	C/T	C/T	C/T	A/G	A/G	C/T	C/T	C/T	A/T	A/G	C/T	C/T	
3	A	A	A	**T**	**C**	T	T	C	G	A	C	T	C	T	G	T	T	
4	A/G	A/G	A/G	**G/T**	**C/T**	C/T	C/T	C/T	A/G	A/G	C/T	C/T	C/T	A/T	A/G	C/T	C/T	
5	A/G	A/G	A/G	**G/T**	**C/T**	C/T	C/T	C/T	A/G	A/G	C/T	C/T	C/T	A/T	A/G	C/T	C/T	
6	A	A	A	**T**	**C**	T	T	C	G	A	C	T	C	T	G	T	T	
7	A	A	A	**T**	**C**	T	T	C	G	A	C	T	C	T	G	T	T	
8	A	A	A	**T**	**C**	T	T	C	G	A	C	T	C	T	G	T	T	
9	A/G	A/G	A/G	**G/T**	**C/T**	C/T	C/T	C/T	A/G	A/G	C/T	C/T	C/T	A/T	A/G	C/T	C/T	
10	A/G	A/G	A/G	**G/T**	**C/T**	C/T	C/T	C/T	A/G	A/G	C/T	C/T	C/T	A/T	A/G	C/T	C/T	
C. pulchellum																		
1	A	A	A	**T**	**C**	T	T	C	G	A	C	T	C	T	G	T	T	
2	A/G	A/G	A/G	**G/T**	**C/T**	C/T	C/T	C/T	A/G	A/G	C/T	C/T	C/T	A/T	A/G	C/T	C/T	
3	A/G	A/G	A/G	**G/T**	**C/T**	C/T	C/T	C/T	A/G	A/G	C/T	C/T	C/T	A/T	A/G	C/T	C/T	

	CoxA					***OmpA***	
	624 bp (0/5)					**242 bp (2/2)**	
ID	Variable sites						
	104	143	246	264	311	**64**	**235**
C. puella							
1	A	A	C	C	G	**G**	**A**

2	A/G	A/G	C/T	C/T	A/G	**A/G**	**A/G**
3	A	A	C	C	G	**G**	**A**
4	A/G	A/G	C/T	C/T	A/G	**A/G**	**A/G**
5	A/G	A/G	C/T	C/T	A/G	**A/G**	**A/G**
6	A	A	C	C	G	**G**	**A**
7	A	A	C	C	G	**G**	**A**
8	A	A	C	C	G	**G**	**A**
9	A/G	A/G	C/T	C/T	A/G	**A/G**	**A/G**
10	A/G	A/G	C/T	C/T	A/G	**A/G**	**A/G**
C. pulchellum							
1	A	A	C	C	G	**G**	**A**
2	A/G	A/G	C/T	C/T	A/G	**A/G**	**A/G**
3	A/G	A/G	C/T	C/T	A/G	**A/G**	**A/G**



Appendix Figure S1: FISH images of bed bugs ovaries. **A**: Tropharium portion of *Rickettsia*-infected line. Rectangle indicated the enlarged field showing in **B. B:** Upper layer of z-stacks from the enlarged field of **A** showing nucleus (n) of a bacteriocyte (bc) with the presence of the three symbionts; *Wolbachia* (green), BEV-like symbiont (yellow) and *Rickettsia* (red). **C**: Tropharium portion of *Rickettsia*-free line. Rectangle indicated the enlarged field showing in **D. D:** Upper layer of z-stacks from the enlarged field of **C** showing nucleus of a bacteriocyte with the presence of only *Wolbachia* and BEV-like symbiont.